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**MICROBIOLOGICAL METHODS
FOR THE
WATER RECOVERY SYSTEMS TEST**

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Microbiological Methods for the Water Recovery Systems Test

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MICROBIOLOGICAL METHODS FOR THE WATER RECOVERY SYSTEMS TEST

1.0 INTRODUCTION

Current microbiological parameters specified to verify microbiological quality of Space Station Freedom water quality include the enumeration of total bacteria, anaerobes, aerobes, yeasts and molds, enteric bacteria, gram positives, gram negatives and *E. coli*. In addition, other parameters have been identified as necessary to support the Water Recovery Test activities to be conducted at the NASA/MSFC later this year. These include: aerotolerant eutrophic mesophiles, legionellae and an additional method for heterotrophic bacteria.

If inter-laboratory data are to be compared to evaluate quality, analytical methods must be eliminated as a variable. Therefore, each participating laboratory must utilize the same analytical methods and procedures. Without this standardization, data can be neither compared or validated between laboratories.

Multiple laboratory participation represents a conservative approach to insure quality and completeness of data. Invariably, sample loss will occur in transport and analyses. Natural variance is a reality on any test of this magnitude and is further enhanced because biological entities, capable of growth and death, are specific parameters of interest. The large variation due to the participation of human test subjects has been noted with previous testing. The resultant data might be dismissed as "out of control" unless intra-laboratory control is included as part of the method or if participating laboratories are not available for verification.

The purpose of this document is to provide standardized laboratory procedures for the enumeration of certain microorganisms in water and wastewater specific to the water recovery systems test. At the present time, the document consists of ten separate cultural methods and one direct count procedure. It is not intended nor is it implied to be a complete microbiological methods manual.

2.0 ENUMERATION PROCEDURES

2.1 Membrane Filtration Method

Media preparation. Media for this test is prepared by dispensing 5 mL of sterile molten media into 50 or 60 mm diameter sterile petri dishes. See the appropriate method in the following sections.

Sterilization of apparatus and materials. All glassware, filter units, filter holders and utensils are presterilized at 121 degrees Celsius for 15 minutes.

Membrane filters. 47 mm diameter, 0.45 um porosity, white gridded (Millipore Corporation, Bedford, MA) membranes are used for this procedure except where indicated otherwise by a specific method. These filters should be purchased presterilized.

Volume requirements. When less than 20 mL of sample (including diluent) is to be filtered, add approximately 10 mL of sterile buffered dilution water to the funnel before filtration. Unequal distribution of bacteria may occur if less water is filtered. Plates which lack uniform distribution cannot be counted.

The membrane filtration test has a range of limits for colonies per plate. Depending on the specific parameters of interest, acceptable countable ranges are between 20-60 or 20-80 colonies per filter.

Dilution and Rinse Water. Sterile peptone dilution and rinse water will be used for all dilution and rinse procedures except where specifically noted. A 10% peptone water stock solution is prepared and autoclaved. The dilution/rinse water is prepared by diluting a measured volume of the sterile stock solution to a final concentration of 0.1%. The final pH is adjusted to 6.8 ± 0.2 at 25°C. The dilution water is dispensed to provide 99 ± 2 mL after autoclaving at 121°C for 15 minutes. The rinse water is dispensed in quantities convenient for handling (approximately 1 L). The prepared rinse water is autoclaved at 121°C for 20 minutes prior to storage and/or use. All peptone dilution and rinse water must be checked for sterility prior to use. Any bottles demonstrating turbidity should be discarded. If more than 10% of the bottles are contaminated the entire batch must be discarded. Record these results in the media preparation log book.

Sample aliquots. Duplicate aliquots of 100 mL (or the maximum filterable volume), 10 and 1 mL are filtered for each sample. Duplicates for sample dilutions of 0.1, 0.01 and 0.001 mL are also prepared and filtered. The filters are then aseptically transferred to plates containing the appropriate medium.

Samples containing urine pretreatment and/ or brine mixtures should be treated prior to membrane filtration. This will reduce the possible inhibition or damage of microorganisms on the filter surface due to the low pH and/or ionic concentration of these samples. Sample volumes of 10 and 1 mL are added directly to 99 mL of sterile phosphate buffer (Section 2.2.1), shaken as recommended, and the entire contents filtered. Sample dilutions of 0.1, 0.01 and 0.001 mL are also prepared using sterile phosphate buffer and filtered as above. Rinse funnels as described in Section 2.1 substituting sterile phosphate buffered water for peptone water. Sample volumes exceeding 10 mL cannot be analyzed using this procedure.

Quality control. Prepare at least two replicate plates for each sample aliquot and dilution used. Insert a sterile dilution water blank (99 mL) at the start of each sample analysis to check for contamination. Record the results on the appropriate worksheet.

Incubation. Incubate the plates inverted as directed in the specified procedure used.

Interpretation of results. Count the plates as directed in the appropriate procedure using a Quebec type colony counter. Do not use electronic counting probes to count colonies since many of the isolates must be subsequently identified. The results are then recorded on the appropriate Worksheet.

If counts from all membrane filters are zero, calculate the number of colonies per 100 mL that would have been reported if there had been one colony on the filter representing the largest filtration volume for that sample. Report as less than (<) that number of colonies per 100 mL.

If colonies are too numerous to count, use the upper limit count from the smallest filtration volume for that sample. Report as greater than (>) that number per 100 mL.

If there is no result because of confluency, lab accident, etc., report as "No Result" and specify reason.

Counts obtained from the sterile dilution blank must be less than or equal to 1 CFU per plate. If the number exceeds this limit the entire sample analysis is labeled as "suspect" and appropriate action is taken to identify and resolve the problem.

Successive filtrations. Decontaminate the funnels between successive filtrations by using an ultraviolet sterilizer. Irradiate 2 minutes with the funnel upside down, then invert and irradiate 3 minutes. Filtration equipment should be autoclaved between filtration series. A filtration series ends when 30 minutes elapse between successive filtrations or every ten (10) samples.

Do not expose the medium or membrane filter culture preparations to random UV radiation leaks that might emanate from the sterilization cabinet. Eye protection is recommended.

Procedure

- 1) Aseptically place a sterile membrane filter over porous plate of receptacle. Carefully place the funnel unit over receptacle and lock it in place.
- 2) Shake sample bottle vigorously (approximately 25 times in 7 seconds) to evenly distribute the bacteria. Take care to secure the screw-cap to prevent leakage during shaking.
- 3) Filter sample under partial vacuum (≤ 10 psi). Higher vacuum may result in cell damage/death and erroneous counts.
- 4) With filter still in place, rinse funnel by filtering three 20-30 mL portions of sterile peptone rinse water.
- 5) Unlock and remove the funnel, immediately remove membrane filter with sterile forceps, and place it on sterile pad or agar with a rolling motion to avoid entrapment of air.

2.1.1 Enumeration of Aerotolerant Heterotrophic Bacteria Using R2A Agar

This procedure is designed for the isolation and enumeration of heterotrophic bacteria from water samples containing low concentrations of organic carbon. The medium used, R2A, is a low nutrient medium designed to culture heterotrophic bacteria. This includes oligotrophic bacteria characteristically found in these aquatic environments.

Procedure

Preparation. Suspend 18.2 grams of Bacto R2A medium (Difco Laboratories, Detroit, MI) in 1 liter of deionized or distilled water. The final pH of the medium should be 7.2 ± 0.2 at 25 degrees Celsius. If necessary adjust the pH using solid K_2HPO_4 or KH_2PO_4 prior to boiling.

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the agar, then promptly remove the medium from the heat.

Sterilization. Autoclave at 121 degrees Celsius for 15 minutes. Cool medium to 50 degrees Celsius in a preheated water bath. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

Dispensing. Within a Laminar Flow hood, aseptically dispense approximately 5 mL of the sterile medium into sterile 50 or 60 mm diameter Petri dishes.

Allow the agar to solidify and cool in the laminar flow hood with the lids slightly ajar to prevent the excess accumulation of moisture in the plates.

Quality control. The prepared medium should be light amber in color and is translucent. Darkened medium may indicate an increased time or temperature in sterilization and should not be used.

Allow plates to incubate at room temperature, in the dark for 24 hours and inspect for contamination prior to use. Record the number of contaminated plates in the Media Preparation Log Book. Discard all contaminated plates. If more than 10% of the plates are contaminated, discard the entire batch.

Prepare a positive control using Staphylococcus epidermidis (ATCC 12228). The resultant colonies should appear small, round and smooth with a creamy white color. Record the results in the media preparation log book. This is not a selective medium, therefore a negative control is not required.

Storage. The prepared plates may be stored at 4 degrees Celsius in sealed bags for up to 14 days. Plates should be stored in the dark and allowed to equilibrate to room temperature just prior to use.

Membrane filters. See Section 2.1.

Sample application. Follow the Membrane Filtration Method described in Section 2.1.

Incubation. Incubate the cultures aerobically at 28 ± 0.5 degrees Celsius for 7 days. Examine the plates after 24 and 48 hours and again after 7 days as some oligotrophic bacteria may require longer incubation periods. Count the colonies at the time of each observation.

Interpretation of results. There may be a variety of colony morphologies and pigmentation as this procedure will culture many bacterial types. Count all colonies present. The countable range of colonies is between 20-80 colonies per plate. Plates should be counted using the magnifying lens of a Quebec type colony counter.

Confirmation. All colony types but a minimum of 10 isolates will be identified.

2.1.2 Enumeration of Aerotolerant Heterotrophic Bacteria using Plate Count Agar

This procedure is designed for the isolation and enumeration of heterotrophic bacteria from water and wastewater samples by membrane filtration. The medium used, Plate Count Agar (PCA), is a non-selective nutrient medium designed to culture heterotrophic bacteria. This medium will 1) provide an excellent medium for the isolation of copiotrophic bacteria, 2) provide a continuum for comparison of CMIF test data with previous subsystem tests and 3) allow for correlation of the Gram positive, Gram negative and enteric procedures.

Procedure

Preparation. Suspend 8.5 grams of Bacto m-Plate Count Broth and 10 grams of Bacto Agar (Difco Laboratories, Detroit, MI) in 1 liter of deionized or distilled water.

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the agar, then promptly remove the medium from the heat. The final pH of the medium should be 7.0 ± 0.2 at 25 degrees Celsius.

Sterilization. Autoclave at 121 degrees Celsius for 15 minutes. Cool medium to 50 degrees Celsius in a preheated water bath. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

Dispensing. Within a Laminar Flow hood, aseptically dispense 5 mL of the sterile medium into sterile 50 or 60 mm diameter Petri dishes.

Allow the agar to solidify and cool in the laminar flow hood with the lids slightly ajar to prevent the excess accumulation of moisture in the plates.

Quality control. The prepared medium should be light amber in color and slightly opalescent. Darkened medium may indicate an increased time or temperature in sterilization and must not be used.

Allow plates to incubate at room temperature, in the dark for 24 hours and inspect for contamination prior to use. Record the number of contaminated plates in the Media Preparation Log Book. Discard all contaminated plates. If more than 10% of the plates are contaminated, discard the entire batch.

Prepare a positive control using Staphylococcus epidermidis (ATCC 12228). The resultant colonies should appear small, round and smooth with a creamy white color. Record the results in the media preparation log book. This is not a selective medium, therefore a negative control is not required.

Storage. The prepared plates may be stored at 4 degrees Celsius in sealed bags for up to 14 days. Plates should be stored in the dark and allowed to equilibrate to room temperature just prior to use.

Membrane filters. See Section 2.1.

Sample application. Follow the Membrane Filtration Method described in Section 2.1.

Incubation. Incubate the cultures aerobically at 28 ± 0.5 degrees Celsius for 7 days. Examine the plates after 24 and 48 hours and again after 7 days as some heterotrophic bacteria may require long incubation periods. Count the colonies at the time of each observation.

Interpretation of results. There may be a variety of colony morphologies and pigmentation as this procedure will culture many bacterial types. Count all colonies present. The countable range of colonies is between 20-80 colonies per plate. Plates should be counted using the magnifying lens of a Quebec type colony counter.

Confirmation. All colony types but a minimum of 10 isolates will be identified.

2.1.3 Enumeration of Aerotolerant Eutrophic Mesophiles

This procedure is designed for the isolation and enumeration of bacteria associated with the human body. This includes normal flora, opportunistic and pathogenic bacteria. Fastidious organisms with a variety of growth requirements will grow on this medium. The hemoglobin component of the chocolate agar provides iron and the supplement VX provides preformed factors which are essential for the culture of some fastidious organisms associated with the normal human flora.

Procedure

Preparation. Suspend 71 grams of Bacto Chocolate Enriched Agar (Difco Laboratories, Detroit, MI) in 1 liter of deionized or distilled water.

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the agar, then promptly remove the medium from the heat. Final pH of the medium should be 7.2 ± 0.2 at 25°C .

Sterilization. Autoclave at 121 degrees Celsius for 15 minutes. Cool medium to 50 degrees Celsius in a preheated water bath. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

Aseptically add the quantity of medium supplement as directed. Bacto supplement VX is presterilized and heat-labile. Return the flask to the magnetic stirrer and mix so that the VX supplement is homogeneously dispersed throughout the medium.

Dispensing. Within a Laminar Flow hood, aseptically dispense 5 mL of the sterile medium into sterile 50 or 60 mm diameter Petri dishes.

Allow the agar to solidify and cool in the laminar flow hood with the lids slightly ajar to prevent the excess accumulation of moisture in the plates.

Quality control. The prepared medium should appear chocolate brown in color.

Allow plates to incubate at room temperature, in the dark for 24 hours and inspect for contamination prior to use. Record the number of contaminated plates in the Media Preparation Log Book. Discard all contaminated plates. If more than 10% of the plates are contaminated, discard the entire batch.

Prepare a positive control using Haemophilus hemolyticus (ATCC 33390). The resultant colonies should appear as small, semi-opaque and be gray-white in color. Record result in the media preparation log book. This is not a selective medium, therefore a negative control is not required.

Storage. The prepared plates may be stored at 4 degrees Celsius in sealed bags for up to 14 days. Plates should be stored in the dark and allowed to equilibrate to room temperature just prior to use.

Membrane filters. See Section 2.1.

Sample application. Follow the Membrane Filtration Method described in section 2.1.

Incubation. Incubate the cultures aerobically under an increased CO₂ tension of 5% and relative humidity of 90%, at 35 ± 0.5 degrees Celsius for 48 hours.

Interpretation of results. Examine plates and record results after 24 and 48 hours incubation. Magnification may be required to detect small opaque to transparent colonies possibly representing fastidious human pathogens. Count all colonies present on the plate. The countable range of colonies is between 20-80 colonies per plate. Plates should be counted using the magnifying lens of a Quebec type colony counter.

Confirmation. All colony types but a minimum of 10 isolates will be identified.

2.1.4 Enumeration of Gram Negative Bacteria

This procedure is designed for the enumeration of Gram negative bacteria from a water sample using the membrane filtration technique. The medium consists of a tryptose, glucose, yeast extract Agar base to enrich for the target organisms and crystal violet to selectively inhibit the growth of Gram positive bacteria.

Procedure

Preparation. Suspend 8.5 grams of Bacto mPC broth base and 10 grams Bacto Agar (Difco Laboratories, Detroit, MI) in 1 liter of deionized or distilled water. Add 1 mL of a stock solution containing 400 mg Bacto crystal violet/ 100 mL making a final concentration of 4 mg/liter (1:250,000).

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the agar, then promptly remove the medium from the heat. The final pH of the medium should be 7.0 ± 0.2 at 25 degrees Celsius.

Sterilization. Autoclave at 121 degrees Celsius for 15 minutes. Cool medium to 50 degrees Celsius in a preheated water bath. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

Dispensing. Within a Laminar Flow hood, aseptically dispense 5 mL of the sterile medium into sterile 50 or 60 mm diameter Petri dishes.

Allow the agar to solidify and cool in the laminar flow hood with the lids slightly ajar to prevent the excess accumulation of moisture in the plates.

Quality control. The modified Plate Count Agar with Crystal Violet should appear purple and be clear to slightly opalescent.

Allow plates to incubate at room temperature, in the dark for 24 hours and inspect for contamination prior to usage. Record the number of contaminated plates in the Media Preparation Log Book. Discard all contaminated plates. If more than 10% of the plates are contaminated, discard the entire batch.

As a positive control, streak a randomly selected plate using Escherichia coli (ATCC 25922). The resultant colonies should be umbonate in elevation, have a rough surface and edge, and be white to purple in color. As a negative control, streak a second randomly selected plate with Staphylococcus epidermidis (ATCC 12228). There should be little to no growth on this plate. Record the results in the media preparation log book.

Storage. The prepared plates may be stored at . degrees Celsius in sealed bags for up to 14 days. Plates should be stored in the dark and allowed to equilibrate to room temperature just prior to use.

Membrane filters. See Section 2.1.

Sample application. Follow the Membrane Filtration Method described in section 2.1.

Incubation. Incubate the cultures aerobically at 28 ± 0.5 degrees Celsius for 7 days (see Method 2.1.2).

Interpretation of results. Examine the plates and record results after 24 and 48 hours incubation and then at 7 days. Pinpoint Gram positive colonies may appear on the plate surface. Compare these colonies to any colonies that may be present on the negative control plate. Disregard those colonies as Gram positive. Count remaining colonies as Gram negative. The countable range of colonies is between 20-80 colonies per plate.

Confirmation. Pick a minimum of 10 suspected Gram negative colonies from the countable plates and perform Gram stains. Gram negative bacteria characteristically stain pink to red.

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2.1.5 Enumeration of Gram Positive Bacteria

This procedure employs the use of a selective medium for the enumeration of Gram positive bacteria from water samples using the membrane filtration technique. The addition of phenylethanol to a nutritional medium will permit the growth of Gram positive organisms but will inhibit the growth of most Gram negative organisms from a mixed sample.

Procedure

Preparation. Suspend 8.5 grams of Bacto MPC broth medium, 10 grams Bacto Agar (Difco Laboratories, Detroit, MI) and 2.5 grams phenylethanol in 1 liter of deionized or distilled water.

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the agar, then promptly remove the medium from the heat. The final pH of the medium should be 7.0 ± 0.2 at 25 degrees Celsius.

Sterilization. Autoclave at 121 degrees Celsius for 15 minutes. Cool medium to 50 degrees Celsius in a preheated water bath. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

Dispensing. Within a Laminar Flow hood, aseptically dispense 5 mL of the sterile medium into sterile 50 or 60 mm diameter Petri dishes.

Allow the agar to solidify and cool in the laminar flow hood with the lids slightly ajar to prevent the excess accumulation of moisture in the plates.

Quality control. The modified PCA with phenylethanol should appear light amber in color and is slightly opalescent.

Allow plates to incubate at room temperature, in the dark for 24 hours and inspect for contamination prior to usage. Record the number of contaminated plates in the Media Preparation Log Book. Discard all contaminated plates. If more than 10% of the plates are contaminated, discard the entire batch.

Prepare a positive control using Staphylococcus epidermidis (ATCC 12228). The resultant colonies should appear small, round and smooth with a creamy white color. Streak a second randomly selected plate with Escherichia coli (ATCC 25922). This plate serves as the negative control. There should be little to no growth on this plate. Record the results for each batch in the media preparation log book.

Storage. The prepared plates may be stored at 4 degrees Celsius in sealed bags for up to 14 days. Plates should be stored in the dark and allowed to equilibrate to room temperature just prior to use.

Membrane filters. See Section 2.1.

Sample application. Follow the Membrane Filtration Method described in Section 2.1.

Incubation. Incubate the cultures aerobically at 28 ± 0.5 degrees Celsius for 7 days (see Method 2.1.2).

Interpretation of results. Examine plates and record the results after 24 and 48 hours and at 7 days. Pinpoint Gram negative colonies may appear on the plate surface. Compare these colonies to any colonies that may be present on the negative control plate. Disregard those colonies as Gram negative. Count remaining colonies as Gram positive. The countable range of colonies is between 20-80 colonies per plate.

Confirmation. Pick a minimum of 10 suspected Gram positive colonies from the countable plates and perform Gram stains. Gram positive bacteria characteristically stain purple.

2.1.6 Enumeration of Yeasts and Molds

This procedure is designed for the enumeration of yeasts and molds. The medium (Emmons) consists of a low nutrient base with an anti-bacterial agent. In addition, the incorporation of Rose Bengal into the medium will further suppress the growth of bacteria and limit the size and height of mold colonies.

Procedure

Preparation. Suspend 30 grams Bacto Sabouraud Dextrose Broth and 10 grams agar in 1 liter of distilled or deionized water. Add 0.05 grams rose bengal and heat with continuous stirring allowing the medium to boil in order to completely dissolve the agar. Promptly remove the medium from the heat when it boils. The final pH of the medium should be adjusted to 7.0 ± 0.2 at 25°C using 1N NaOH.

Note: Rose bengal is added to this medium as a selective agent for some bacteria and to restrict the size of mold colonies due to the increased incubation time. Some researchers have noted that rose bengal may deteriorate to inhibitory products by UV irradiation. Plates should be stored in the dark until just prior to use.

Sterilization. Autoclave at 121 degrees Celsius for 15 minutes. Cool medium to 50 degrees Celsius in a preheated water bath. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

Antibiotic addition. Prepare the Chloramphenicol by aseptically adding 2 ml ethanol to Bacto Antimicrobial Supplement C. Add entire contents of the rehydrated vial to the cooled medium.
Note: Do not use vials that have been rehydrated for more than 24 hours. Store vials refrigerated in the dark.

Dispensing. Within a Laminar Flow hood, aseptically dispense 5 mL of the sterile medium into sterile 50 or 60 mm diameter Petri dishes.

Allow the agar to solidify and cool in the laminar flow hood with the lids slightly ajar to prevent the excess accumulation of moisture in the plates.

Quality control. The dehydrated Modified Sabouraud Dextrose Agar should appear light beige. Prepared medium will appear amber to light pink and will be opalescent. Record the result in the Media Preparation Log Book.

Streak a randomly selected plate using Saccharomyces cerevisiae (ATCC 9763) as a positive control. The resultant colonies should appear round, smooth and be pink to red. Prepare a negative control using Xanthomonas maltophilia (ATCC 13637). There should be little to no growth on this plate. Record the results for each batch in the media preparation log book.

Storage. The prepared plates may be stored at 4 degrees Celsius in sealed bags for up to 14 days. Plates should be stored in the dark and allowed to equilibrate to room temperature just prior to use.

Membrane filters. See Section 2.1.

Sample application. Follow the Membrane Filtration Method described in Section 2.1.

Incubation. Incubate the plates aerobically at 20 ± 0.5 degrees Celsius for 5 days. Examine the plates and record the results after 48 hours and then daily.

Interpretation of results. Yeast colonies will have a smooth surface and color may vary. Mold colonies will have rough to downy appearance with a variety of colors. Colonies may discolor the medium slightly.

The acceptable countable range of colonies is between 20-60 colonies per plate.

Confirmation. Confirm molds by colonial morphology under binocular dissecting scope (10X). Yeast may be confirmed based on cellular morphology.

2.1.7 Enumeration of Legionellae

This procedure is designed for the isolation and enumeration of Legionella spp. This method relies on membrane filtration for the initial concentration of legionellae from samples. The filters are subsequently acid treated and aliquots plated directly on a selective enriched agar using the spread plate technique. The BBL BCYE Agar base (Baltimore Biological Laboratories, Cockeysville, MD) consists of a basal medium containing ACES buffer, charcoal, ferric pyrophosphate (0.25 g/L) and alpha-ketoglutarate. A special lyophilized enrichment containing L-cysteine (0.4 g/L) is added. Legionellae are fastidious bacteria which are easily overgrown by other bacteria in the environment. As a precaution against this, an acid treatment step is used to reduce the numbers of competing bacteria. A mixture of antibiotics (BBL PAV supplement) which contains Polymyxin B (100,000 units), Vancomycin (5 mg) and Anisomycin (80 mg) is added to each liter of medium to further minimize overgrowth of unwanted bacteria.

Note: Special precaution should be taken when working with any sample suspected of containing Legionellae as all species are potential human pathogens. This includes specific precautions to prevent aerosol formation.

Procedure

Preparation. Suspend 38.3 grams of BBL BCYE agar base (BBL Cockeysville, MD) and 3 grams glycine in 900 mL of deionized or distilled water. The final pH of the medium should be 6.9 ± 0.2 at 25° C. If necessary adjust the pH using 1N KOH. Do not use NaOH since legionellae are sensitive to free sodium ions. Care should be taken to adjust the pH of the medium in order to obtain optimal recovery. After adjusting the pH, bring the volume of the medium to 1 L using distilled or deionized water.

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the agar, then promptly remove the medium from the heat.

Sterilization. Autoclave at 121 degrees Celsius for 15 minutes. Cool medium to 50 degrees Celsius in a preheated water bath. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

Enrichment and antibiotic addition. Aseptically rehydrate and add a vial of the BBL cysteine (0.4 g/10 mL) supplement using sterile distilled or deionized water per liter of prepared and cooled media. Also rehydrate and add a vial of BBL PAV antibiotic supplement using sterile distilled or deionized water per liter of prepared and cooled media.

Dispensing. Withi. a Laminar Flow hood, aseptically dispense the sterile medium into sterile 15 x 100 mm Petri dishes. Continually agitate the flask while pouring plates to keep charcoal from settling out of the media. Allow the agar to solidify and cool in the laminar flow hood with the lids slightly ajar to prevent the excess accumulation of moisture in the plates.

Quality control. The prepared BBL BCYE Agar should appear gray-black in color.

Allow plates to incubate at room temperature, in the dark for 24 hours and inspect for contamination prior to usage. Record the number of contaminated plates in the Media Preparation Log Book. Discard all contaminated plates. If more than 10% of the plates are contaminated, discard the entire batch.

Prepare a positive control using Legionella gormanii (ATCC 33297). Colonies of Legionella spp. should be visible after 2 to 3 days incubation and appear light blue to blue-gray in color and should not discolor medium. Older colonies will become larger, smoother and gray-white in color. Typical cultural response of Legionella should be evident after 48-72 hours. Prepare a negative control using Escherichia coli (ATCC 25922). This control organism should grow but not produce a blue pigment. Record the results obtained from each batch in the media preparation log book.

Storage. The prepared plates may be stored at 4 degrees Celsius in sealed bags for up to 14 days. Plates should be stored in the dark and allowed to equilibrate to room temperature just prior to use.

Sample preparation. Concentrate maximum amount of water (up to 100 mL), in duplicate, through a 47 mm white, ungridded Nuclepore (Nuclepore Corporation, Pleasanton, CA) membrane having a pore size of 0.2 μ m. Multiple membranes may be used, if necessary, and combined to obtain appropriate concentration. Following concentration place filter(s), soiled side down, in a sterile 50 mL centrifuge tube (or similar vessel) with a screw cap containing ten (10) mL of sterile water. Disperse bacteria from filter by vortexing (3 x 30 seconds) or place in a sonic bath for 10 minutes. Repeat the above procedure concentrating duplicate 10 mL aliquots.

Acid treatment. Place 1 mL of each suspension in a sterile 13 x 100 mm screw cap tube containing 1 mL acid treatment reagent (LATR) and vortex for 10 seconds. Let stand for 15 minutes at room temperature and immediately neutralize by adding 1 mL of the alkaline neutralizing reagent (LANR) and vortex for 10 seconds.

Acid treatment reagent:

Solution A: 0.2M KCl (14.9 g/L in distilled or deionized water)
Solution B: 0.2M HCl (16.7 mL/L 10N HCl in distilled/deionized water)

To prepare: Mix 18 parts solution A with 1 part solution B. pH of this solution should be 2.2 at 25° C. Check pH against a pH 2 standard buffer. Dispense in 1 mL volumes into a 13 x 100 mm screw cap tube and sterilize by autoclaving at 121° C for 15 minutes. Label tubes as Legionella Acid Treatment Reagent (LATR).

Alkaline neutralizer reagent:

Stock solution: 0.1N KOH (6.46 g/L in distilled/deionized water)

To prepare: Dilute 10.7 mL of stock solution using distilled or deionized water to 100 mL. Dispense in 1 mL volumes into a 13 x 100 mm screw cap tubes and sterilize by autoclaving at 121° C for 15 minutes. Label tubes as Legionella Alkaline Neutralizing Reagent (LANR)

Note: Equal volumes of LATR and LANR when mixed should result in a pH of 6.9 at 25° C.

Sample application. Inoculate 0.1 mL of the neutralized acid treated suspensions onto the BBL BCYE supplemented agar plates and spread over the entire surface using a glass rod and turntable (See Section 2.3). Sterilize the rod between plating aliquots by soaking in alcohol and then flaming prior to each use. Also prepare serial dilutions, if necessary, from the 1 mL sample aliquots and plate as described above.

Incubation. Incubate the cultures aerobically in an atmosphere containing 2.5% CO₂ and a relative humidity of 90%, at 35 ± 0.5 degrees Celsius for at least 10 days. Examine daily for evidence of growth and record results.

Interpretation of results. Legionellae colonies will appear blue to grey-blue in color and should not discolor this medium. Any colonies fitting this description should be picked for confirmation. The countable range of colonies is between 30-300 colonies per plate. Determine the number of CFUs/100mL using the formula below:

$$\text{CFUs/100mL} = \frac{333 \times \text{number of colonies}}{\text{volume of sample filtered (mL)}} \times 100$$

Note: Although this medium is designed for the cultivation and isolation of Legionella spp., other organisms may grow and must be differentiated from the target organism. Also, due to the variety of nutritional requirements of the genus, some strains may be encountered that fail to grow or grow poorly on this medium.

Confirmation. All colonies suspected of being Legionella spp. should be Gram stained and subcultured to fresh BCYE Agar plates with and without L-cysteine.

Gram negative organisms that grow on BBL-BCYE agar with cysteine but fail to grow on BBL-BCYE agar without cysteine may be presumptively identified as Legionellae. Definitive identification is performed on all positive presumptive isolates on the basis of growth, morphology, fatty acid profiles and biochemical and immunological reactions.

2.1.8 Enumeration of Enteric Bacteria

This procedure is designed for the isolation and enumeration of enteric bacteria from a water sample using the membrane filtration technique. This will include both Gram positive and Gram negative bacteria which can tolerate the selective action of bile salts. The medium consists of a Tryptose, Glucose, Yeast extract Agar base enrichment with Bile Salts No. 3 to inhibit nonenteric bacteria.

Procedure

Preparation. Suspend 8.5 grams of dehydrated Bacto m-PC broth medium, 10 grams Bacto Agar and 1.5 grams of Bacto Bile Salts No. 3 (Difco Laboratories, Detroit, MI) in 1 L distilled or deionized water.

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the agar, then promptly remove the medium from the heat. The final pH of the medium should be 7.0 ± 0.2 at 25°C .

Sterilization. Autoclave at 121 degrees Celsius for 15 minutes. Cool medium to 50 degrees Celsius in a preheated water bath. Do not allow the medium to stand for more than 1 hour between preparation and autoclaving.

Dispensing. Within a laminar flow hood, aseptically dispense 5 mL of the sterile medium into sterile 50 or 60 mm diameter Petri dishes.

Allow the agar to solidify and cool in the laminar flow hood with the lids slightly ajar to prevent the excess accumulation of moisture in the plates.

Quality control. The modified Plate Count Agar with Bile Salts no. 3 should appear slightly amber in color and is translucent.

Allow plates to incubate at room temperature, in the dark for 24 hours and inspect for contamination prior to usage. Record the number of contaminated plates in the Media Preparation Log Book. Discard all contaminated plates. If more than 10% of the plates are contaminated, discard the entire batch.

Streak a randomly selected positive control plate using Escherichia coli (ATCC 25922). The resultant colonies should be umbonate in elevation, have a rough surface and edge, and be off-white in color. As a negative control, streak a second randomly selected plate with Bacillus alvei (ATCC 6344). Little to no growth should be observed. Record the results obtained from each batch in the media preparation log book.

Storage. The prepared plates may be stored at 4 degrees Celsius in sealed bags for up to 14 days. Plates should be stored in the dark and allowed to equilibrate to room temperature just prior to use.

Membrane filters. See Section 2.1.

Sample application. Follow the Membrane Filtration Method described in section 2.1.

Incubation. Incubate the cultures aerobically at 35 ± 0.5 degrees Celsius for 48 hours.

Interpretation of results. Examine the plates after 24 and 48 hours. Colonies should appear off-white to yellow and should not discolor the medium significantly. Examine plates closely for the presence of small, off-white, translucent colonies that represent fecal Streptococci. Count all colonies. The countable range of colonies with this medium is between 20-80 colonies per plate.

Confirmation. Confirm enteric bacteria by picking at least 10 isolates transferring them to Bacto Lauri Tryptose broth and Enterococci Confirmatory broth. Incubate at 35 degrees Celsius for 24 and 48 hours and examine for growth. Growth in either tube indicates recovery of enteric bacteria.

Note: Lauri Tryptose broth is prepared by dissolving 35.6 grams Bacto Lauri Tryptose broth base in 1 L distilled or deionized water. Enterococci confirmatory broth is prepared by dissolving 80.4 grams of Bacto ECB into 1 L of distilled or deionized water. Each media is dispensed into 15 x 125 mm screw cap tubes in 5 mL quantities and autoclaved at 121°C for 15 minutes.

2.1.9 Enumeration of Fecal Coliform Bacteria

This procedure is designed for the isolation and enumeration of fecal coliforms from a water sample using the membrane filtration technique. The medium consists of a tryptose, lactose, peptone, yeast extract base enrichment with Bile Salts No. 3 to inhibit Gram positive bacteria. Rosolic acid is added for the color reaction of the fecal coliform bacteria. The elevated incubation temperature enhances selectivity and gives 93% accuracy in differentiating between coliforms from warm-blooded animals and those from other sources.

Procedure

Preparation. Suspend 37 grams of dehydrated Bacto mFC Broth Base and 10 grams Bacto Agar (Difco Laboratories, Detroit, MI) in 1 liter of distilled or deionized water. Add 10 mls of a 1% solution of Bacto Rosolic acid in 0.2N NaOH.

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the agar, then promptly remove the medium from the heat. Do not autoclave this medium.

Dispensing. Within a laminar flow hood, aseptically dispense 5 mL of the medium into sterile 50 or 60 mm diameter Petri dishes.

Allow the agar to solidify and cool in the laminar flow hood with the lids slightly ajar to prevent the excess accumulation of moisture in the plates.

Quality Control. The mFC agar should be cranberry red in color and be slightly opalescent. The medium should appear blue before the addition of the Rosolic acid.

Allow plates to incubate at room temperature, in the dark for 24 hours and inspect for contamination prior to usage. Record the number of contaminated plates in the Media Preparation Log Book. Discard all contaminated plates. If more than 10% of the plates are contaminated, discard the entire batch.

Streak a randomly selected plate with Escherichia coli (ATCC 25922) to serve as the positive control. The resultant colonies should be umbonate in elevation, have a rough edge, and be blue in color. Streak a second randomly selected plate using Xanthomonas maltophilia (ATCC 13637). This plate will serve as the negative control plate. The resultant colonies should appear round, smooth and be gray in color. Record the results obtained for each batch in the media preparation log book.

Storage. The prepared plates may be stored at 4 degrees Celsius in sealed bags for up to 14 days. Plates should be stored in the dark and allowed to equilibrate to room temperature just prior to use.

Membrane filters. See Section 2.1.

Sample application. Follow the Membrane Filtration Method described in section 2.1.

Incubation. Place the prepared cultures in watertight plastic bags. Submerge the bags containing plates in a water bath at 44.5 ± 0.2 degrees Celsius for 24 ± 2 hours. The bags should be anchored under the water to maintain the critical temperature requirements. Place all prepared plates containing filters in the water bath within 30 minutes after filtration.

Interpretation of results. Examine the plates after 24 hours. Fecal coliform colonies should appear blue in color and nonfecal colonies should appear gray. Few nonfecal colonies should be present on the plates due to the inhibitory effect of the medium and incubation temperature. The countable range of colonies with this medium is between 20-60 colonies per plate.

Confirmation. Verify fecal coliforms by picking at least 10 isolates exhibiting a blue color and transfer to EC broth. Incubate at 44.5 ± 0.2 degrees Celsius for 24 hours. Confirmation of fecal coliforms is indicated by gas accumulation in the Durham tube.

Note: EC broth is prepared by dissolving 37 grams Bacto EC broth base in 1 L of distilled or deionized water. The medium is brought to a boil with continuous stirring, dispensed into 15 x 125 mm screw cap tubes in five mL quantities and autoclaved at 121°C for 15 minutes.

2.1.10 Enumeration Of Anaerobic Bacteria

This procedure is designed for the cultivation and enumeration of anaerobic bacteria from a water sample using the membrane filtration technique. The medium consists of a nutritious base and thioglycolate which helps insure a reduced anaerobic medium.

Procedure

Preparation. Suspend 58 grams of Difco Brewer Anaerobic Agar in 1 liter of oxygen free deionized or distilled water.

Heat with continuous stirring allowing the medium to boil in order to completely dissolve the agar, then promptly remove the medium from the heat. The final pH of the medium should be 7.2 ± 0.2 at 25°C .

Note: Oxygen free water may be prepared by boiling the water for five minutes prior to use. Care should be taken that a sufficient quantity of water is allowed to boil to account for loss due to evaporation during boiling.

Sterilization. Autoclave at 121 degrees Celsius for 15 minutes. Cool medium to 50 degrees Celsius in a preheated water bath. Do not allow the medium to stand for more than 15 minutes between preparation and autoclaving. Do not allow the medium to stand for more than 1 hour at 50°C before pouring plates.

Dispensing. Within a Laminar Flow hood, aseptically dispense 5 mL of the sterile medium into sterile 50 or 60 mm diameter x 9 mm Petri dishes.

Allow the agar to solidify and cool in the laminar flow hood with the lids slightly ajar to prevent the excess accumulation of moisture in the plates.

Plates poured should be stored immediately upon solidification for 1-2 days in the reduced atmosphere of the anaerobe chamber or anaerobe jar prior to use.

Quality control. The prepared medium should initially appear beige becoming red in color due to aeration upon standing. Do not use any plates which have turned red.

Allow plates to incubate at room temperature, in the dark for 24 hours under anaerobic conditions and inspect for contamination prior to use. Record the number of contaminated plates in the Media Preparation Log Book. Discard all contaminated plates. If more than 10% of the plates are contaminated, discard the entire batch.

Streak a randomly selected positive control plate using Clostridium beijerinckii (ATCC 17795) and incubate under anaerobic conditions. The resultant colonies should be circular to irregular, entire to scalloped, flat to raised, translucent, gray in color, shiny and smooth. As a negative control, streak a second randomly selected plate with Xanthamonas maltophilia (ATCC 13637) and incubate under anaerobic conditions. There should be little to no growth on this plate. Record the results obtained for each batch in the media preparation log book.

Storage. The prepared plates may be stored at 4 degrees Celsius under anaerobic conditions for up to 10 days. Plates should be stored in the dark and allowed to equilibrate to room temperature under anaerobic conditions just prior to use.

Membrane filters. See Section 2.1.

Sample application. All work is to be conducted within a reduced oxygen atmosphere. Follow the Membrane Filtration Method described in Section 2.1. Ideally, samples should be processed on-site immediately following collection. A small hand-held type vacuum pump within a nitrogen filled glove bag may be used for filtration purposes. Samples should be processed within two (2) hours following collection. Duplicate samples of 100 mL (or maximum filterable volume), 10 and 1 mL are filtered. Duplicate sample dilutions of 0.1, 0.01 and 0.001 mL are also prepared and filtered. Aseptically transfer the filters to the plates and transport to the laboratory under anaerobic conditions. Note: If samples are transported to a laboratory prior to filtration a transport medium should not be used.

Dilution and rinse water. The dilution and rinse water for filtration purposes will be held for 2 days under an anaerobic atmosphere. If milk dilution bottles are used for diluent water storage then the caps should be loosened so that the contents will outgas any oxygen present.

Incubation. Incubate the cultures anaerobically at 28 ± 0.5 degrees Celsius for 10 days. The anaerobic environment can be produced by the use of a commercially available anaerobe system consisting of an anaerobe jar, anaerobe gas packs, and palladium catalyst. An Anaerobic chamber (glove box) may also be used. The use of an indicator is required to insure achievement and maintenance of a reduced environment.

Interpretation of results. This method will allow the recovery of both anaerobic and facultatively anaerobic bacteria. Examine the plates after 48 hours, 5 days and 10 days. The countable range of colonies with this medium is between 20-60 colonies per plate.

Confirmation. All isolates must be confirmed as strict anaerobes. Colonies are picked and streaked on duplicate Brewers Anaerobic Agar plates. One plate is incubated aerobically and the other plate incubated under anaerobic conditions. Strict anaerobes will not grow on the plate incubated aerobically. All isolates which grow only under anaerobic conditions will be identified.

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2.2 Direct Epifluorescent Filter Technique (DEFT)

The following procedure describes the direct microscopic count of bacteria utilizing a fluorochrome and the membrane filtration technique. This technique is designed to determine the total number of bacteria in water samples.

Procedure

- 1) Select a slide with the appropriate fluorescent stain. Check to see that the proper filter pack is in place in the scope.
- 2) Low fluorescence immersion oil and the 100x objective are used for counting.
- 3) Randomly select a field and count all the bacteria seen in that field, starting at the top of the field, counting left to right. Be sure to count only those objects with "bacterial shapes". Do not count fluorescent debris. Record results.
- 4) Change randomly to another field and count again in the same manner.
- 5) Determine the average number of bacteria per field.
- 6) Determine the total number of fields to count using the following table:

Average number of bacteria per field	Minimum number of fields to count
≥ 15	10
10-14	25
6-9	50
3-5	75
≤ 2	100

- 7) Calculate the total number of bacteria in the water sample (per mL) using the following equations:

$$\text{Total Count} = \text{Mean bacteria count /field} \times \text{Microscopic Factor (MF)}$$

where,

$$\text{MF} = \frac{\text{Area of membrane through which sample is filtered (mm}^2\text{)}}{\text{Microscopic field [or grid] area (mm}^2\text{)} \times \text{Sample volume (ml)}}$$

2.2.1 Acridine Orange Staining For Epifluorescence Microscopy

This procedure utilizes the fluorochrome acridine orange to stain nucleic acids within microbial cells. When coupled with membrane filtration and epifluorescence microscopy, a rapid total direct count of microbial cells in aqueous solutions is feasible.

Sample fixation. Prepare the glutaraldehyde stock fixative solution fresh daily. The fixative stock solution is 5.0% (w/v) glutaraldehyde in phosphate buffer (see composition below). At the time of collection, add the glutaraldehyde fixative solution to the sample equivalent to 10.0% (v/v). The final concentration of glutaraldehyde is 0.5%.

Sample storage. Preserved (fixed) samples may be stored in the refrigerator for up to 21 days.

Procedure

- 1) Prepare fluorochrome as 0.1% (w/v) acridine orange in phosphate buffer. Filter this solution at the time of use through a 0.2 micrometer disposable sterile syringe filter.
- 2) Prepare the phosphate buffer for use in this procedure by dissolving 13.6 g KH_2PO_4 in distilled or deionized water and dilute to 1 liter. Adjust to pH 7.2 if necessary. Filter through a 0.2 micrometer filter. Filter buffer daily before use.
- 3) Use a 25 mm cellulose backing-filter (Millipore Corporation, Bedford, MA) having a 0.45 micrometer porosity. Dampen this filter first with filtered rinse water so that it adheres to the filtration base. Place a Nuclepore (Nuclepore Corporation, Pleasanton, CA) pre-stained (black) polycarbonate filter, 25 mm diameter having a 0.2 micrometer porosity on top of the backing-filter. Assemble the filtration apparatus.
- 4) Determine the volume of sample to be filtered that is required for direct counting. Twenty milliliters of clean or potable water is usually sufficient. Volumes up to 25 ml may be added directly to the filter within the apparatus. If dilutions need to be made use phosphate buffer. For larger volume requirements 25 mL aliquots may be consecutively added and filtered through the same filter.
- 5) Add Acridine Orange stock solution to the sample (or dilution) at a ratio of 1:1 (v/v).
- 6) Stain for 2 minutes.
- 7) Add phosphate buffer to the stain/sample equivalent to a final ratio of 3:1:1 (3 parts buffer to 1 part stain to 1 part sample).
- 8) Filter with vacuum (approximately 13 kPa).

- 9) Rinse with a volume of phosphate buffer equivalent to one half the total volume of the: stain solution + sample + buffer.
- 10) Filter with vacuum (approximately 13 kPa).
- 11) Remove the Nuclepore filter from base and backing-filter by its edge and air dry.
- 12) Place a small drop of low fluorescence immersion oil on a clean slide. Place the filter on the slide so that the sample side faces the objective lens. Add a small drop of oil to the filter and overlay with coverslip.
- 13) Examine filter surface with an epifluorescence microscope and oil immersion objective utilizing low fluorescence oil.
- 14) Determine average number of cells per field and calculate the number of cells/100 mL as specified in Procedure 2.2.

2.3 Direct Enumeration Using the Spread Plate Technique

Procedure

1) Label petri dishes with the sample ID, date, dilution, analyst's initials and any other pertinent information as specified in the laboratories' SOP document. Media type and batch number should have previously been recorded on the plates.

Note: The plated media must be predried. This may be accomplished by incubating the plates with lids on at 55° C for 12-18 hours. A 2-3 gram weight loss is not uncommon.

2) Vortex the sample to be plated for 10 seconds to evenly distribute the bacteria.

3) Pipette 0.1 mL of the sample directly onto the agar surface.

4) Using a bent sterile glass rod, distribute the inoculum over the surface of the medium by rotating the dish on a turntable. Allow the inoculum to be completely absorbed into the media before transferring the plates to the incubator. The spreading rods are sterilized between sample applications by soaking in 90% (v/v) ethanol and flaming prior to use.

5) Incubate at the specified temperature and time as dictated by the procedure.

6) Count colonies as prescribed by the specific procedure (see Section 2.1.7). The countable range using the spread plate technique is between 30-300 colonies per plate (15 x 100 mm).

7) Calculate the number of CFUs/100 mL as described by the procedure and dilutions being used (see Section 2.1.7).

Quality Control:

Prepare at least two replicate plates for each sample aliquot and dilution being used.

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ANALYTICAL CONTROL TEST PLAN
FOR THE
WATER RECOVERY SYSTEMS TESTS
REVISION 1.0

**ANALYTICAL CONTROL TEST PLAN
FOR THE
WATER RECOVERY SYSTEMS TESTS**

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March 12, 1990

ANALYTICAL CONTROL TEST PLAN
FOR THE
WATER RECOVERY SYSTEMS TESTS

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1.0 INTRODUCTION

Quantitative and qualitative results reported by the laboratory are very important to the decision-making process. In some cases, they may represent the only basis for deciding between two or more given options or processes. Therefore, it is essential that handling of laboratory samples and analytical operations employed are performed at a deliberate level of conscientious effort. Reporting erroneous results can be worse than reporting no results at all because they lead to faulty interpretations and can result in misinformed decisions¹.

1.1 Scope

The intent of this document is to provide analytical control specifications which will govern future test procedures related to all Phase III activities to be conducted at the National Aeronautics and Space Administration/Marshall Space Flight Center (NASA/MSFC). This document addresses the process which will be used to verify analytical data generated throughout the test period, identify responsibilities of key personnel and participating laboratories, as well as to ensure that approved methodology and procedures are used during the test activities. The purpose of this document is not to outline specifics but is intended to provide a minimum guideline by which sampling protocols, analysis methodologies and laboratory operations should be developed.

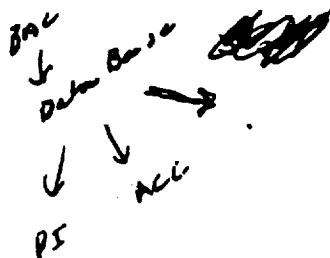
1.2 Analytical Control Coordinator

An Analytical Control Coordinator (ACC) will be appointed by NASA/MSFC to implement and monitor the program described within this document. The ACC will be located on-site at MSFC Building 4755 during sample collection and handling. The ACC will have a minimum of a Master of Science degree, five years experience in quality control/quality assurance and analytical chemistry. In addition, the ACC must possess good written and oral communication skills. The ACC will be responsible for monitoring sample collection activities and preparing blind duplicate and blind reference samples. In addition, the ACC will be responsible for overseeing the splitting of samples to the participating laboratories and will be responsible for implementing and monitoring the interlaboratory verification program.

1.3 Summary

The interlaboratory control verification program will consist of periodic evaluations of laboratory performance and monitoring the quality of the analytical results. Evaluation of laboratory performance will be conducted via the qualification and verification programs described in Section 3.3. The determination of the quality of analytical results will be accomplished using blind reference samples. The results submitted for the blind control samples (included with each batch) will be reviewed by the ACC and the corresponding parameters within that sample batch will be identified as "acceptable" or "suspect" based on these results. The ACC will

report the data directly to the Principal Investigator and Test Conductor. All data relating to test subject safeguards will additionally be released directly to the Medical Monitor by the Principal Investigator.



2.0 APPLICABLE DOCUMENTS

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3.0 REQUIREMENTS FOR THE CHEMICAL ANALYSIS OF WATER

3.1 Sample Collection and Control Procedures

The control of analytical performance begins prior to the actual collection of samples. All control procedures for sample tracking including sample collection, preservation, analysis, storage, and disposal should be in compliance with approved U.S. Environmental Protection Agency (EPA), American Society for Testing and Materials (ASTM) and/or American Public Health Association (APHA) procedures. For specific or more detailed information, consult the sources listed in Section 2.0 of this document. All unusual sample collection techniques required must be written and approved by the MSFC Material and Processing Laboratory and the Principal Investigator prior to use.

3.1.1 Sample Containers

Containers for the collection of various samples will meet or exceed all APHA, ASTM and/or EPA requirements²⁻⁴.

3.1.2 Sample Collection

Sample collection should be accomplished by procedures described by the APHA, EPA unless specified elsewhere^{2,3}. All samples will be collected by analysts trained in aseptic technique. A minimum number of persons should be involved in the actual sampling process. Written procedures will be available on-site for reference and review by sampling personnel. Prior to collection of the actual sample(s) from a specific location at least one hundred milliliters of water will be voided through the collection port. If microbiological samples are to be collected the sample port will be adequately disinfected prior to sample

collection. If both chemical and microbiological samples are to be collected from a given sample location the chemical sample is collected first immediately following initial flushing. Once all the samples are collected for the chemical parameters the port is disinfected and the microbiological samples are collected. Samples to be split to more than one laboratory should be collected as a single sample, thoroughly mixed and then split into the appropriate containers. Samples will be preserved as they are collected as recommended in Section 3.1.4. Sample collection labels should be affixed to each sample container and should minimally contain the following information:

- o Sample collection number
- o Date collected
- o Time collected
- o Collection location/description
- o Initials of personnel collecting the sample
- o Parameters for analysis
- o How preserved
- ~~o Disinfection procedure used~~
- o Any anomalies encountered during sampling
- o Laboratory to which the sample is to be sent

After collection, samples will be delivered to the Data Custodian for weighing and recording into the Master Log. Samples will be handled as little as possible after collection.

3.1.3 Sampling Tracking Procedures

Sample tracking procedures will be maintained for the life cycle of the sample. The sample life cycle will begin when the sample is collected and will continue until final sample disposal. Initial sample tracking will be accomplished using preprinted sample ^{labels} ~~tags~~ and chain of custody documents. Samples will be assigned a Sample Number and logged into a Sample Log Book by the Data Custodian. Subsequent tracking of the

samples will be accomplished as described in Section 6.0.

Each participating laboratory will assign a sample custodian and designate a sample storage area. Incoming samples will be received by the sample custodian and the appropriate chain-of-custody record signed.

Custody of samples within the laboratory is defined as:

- o In actual physical possession of laboratory personnel
- o In view, after being in physical possession
- o In physical possession and in locked storage to prevent tampering
- o In a secured area, restricted to authorized personnel.

If a sample does not meet one of the above categories then it is not in custody⁵. If a sample must leave the primary laboratory, for any reason, the chain of custody form must accompany it.

3.1.4 Sample Preservation and Storage

Sample degradation can begin immediately following collection. Preservation is necessary to retard the degradation of chemicals and/or the alteration of microbial populations in samples prior to analysis. Samples will be processed and relinquished by the Data Custodian within a maximum of six hours after collection. Samples should be run in a timely manner as received by the laboratory. Sample aliquots which have holding times greater than 24 hours and which cannot be analyzed on the day of collection will be preserved and/or stored at 4 degrees Celsius. Table 3.1 illustrates approved preservation, collection and storage containers, as well as acceptable holding times. All sample analyses should be completed within 28 days of laboratory

receipt. Samples not analyzed within the required holding times, samples which have been inadequately preserved or samples otherwise subjected to questionable conditions will be appropriately labeled and this information will be reported to the ACC with the results. The ACC will flag and report these results to the Principal Investigator as "suspect."

All sample preservation will be accomplished at the time of collection. Sample containers will be prepared with the appropriate preservative, sterilized if required, and labeled prior to use.

3.1.5 Sample Transport

Samples requiring transport should be shipped on "blue" ice by an overnight delivery service. "Blue ice" is used so that leakage will not occur and result in courier rejection. All samples requiring the analysis of volatiles must include a travel blank (Section 3.4.3.1.2).

3.1.6 Sample Storage

Samples will be stored by each participating laboratory under proper conditions in a controlled access facility. In addition, representative samples will be frozen and archived by the ACC for later use.

3.1.7 Sample Disposal

Disposal of samples may not occur without prior written consent from the ACC. Following review and analysis of the sample results and corresponding control data, each participating laboratory will be notified of the acceptability of the results. Once results are accepted, the Principal Investigator will

Table 3.1. Recommended Preservation and Holding Times for Water Samples^a

Measurement	Container ^b	Preservative ^{c,d}	Max. Allowable Holding Time ^e
Physical Properties			
Color	P,G	Cool, 4°C	48 Hrs.
Conductance	P,G	Cool, 4°C	28 Days
Dissolved Gas	TBD	TBD	TBD
Hardness	P,G	HNO ₃ to pH<2	28 Days
Odor	G only	Cool, 4°C	24 Hrs.
Particulates	TBD	TBD	TBD
pH	P,G	None Req.	None ^f
Residue			
Filterable	P,G	Cool, 4°C	7 Days
Non-Filterable	P,G	Cool, 4°C	7 Days
Total	P,G	Cool, 4°C	7 Days
Volatile	P,G	Cool, 4°C	7 Days
Settleable Matter	P,G	Cool, 4°C	48 Hrs.
Temperature	P,G	None Req.	None ^f
Turbidity	P,G	Cool, 4°C	48 Hrs.
<u>Metals</u>			
Dissolved	P,G	Filter on site HNO ₃ to pH<2	28 Days — <i>6 mos</i>
Suspended	P,G	Filter on site	28 days ^g — <i>6 mos</i>
Total	P,G	HNO ₃ to pH<2	28 Days — <i>6 mos</i>
Chromium ⁺⁶	P,G	Cool, 4°C	24 Hrs.
Mercury			
Dissolved	P,G	Filter HNO ₃ to pH<2	28 Days
Total	P,G	HNO ₃ to pH<2	48 Hrs.

Table 3.1 (continued)

Inorganics, Non-Metallics

Acidity	P,G	Cool, 4°C	14 Days
Alkalinity	P,G	Cool, 4°C	14 Days
Bromide	P,G	None Req.	28 Days
Chloride	P,G	None Req.	28 Days
Chlorine	P,G	None Req.	None ^f
Cyanides	P,G	Cool, 4°C NaOH to pH>12 0.6g ascorbic acid ^h	14 Days
Fluoride	P,G	None Req.	28 Days
Iodide	P,G	Cool, 4°C	24 Hrs.
Iodine	G only	Cool, 4°C zero head space	None ^f
Nitrogen Ammonia	P,G	Cool, 4°C <i>zero head space</i> H₂SO₄ to pH<2	28 Days <i>18 hours</i> *
Kjeldahl, Total	P,G	Cool, 4°C H ₂ SO ₄ to pH<2	28 Days
Nitrate plus Nitrite	P,G	Cool, 4°C H ₂ SO ₄ to pH<2	28 Days
Nitrate ⁱ	P,G	Cool, 4°C	24 Hrs.
Nitrite	P,G	Cool, 4°C	48 Hrs.
Dissolved Oxygen Probe	G bottle and top	None Req.	None ^f
Winkler	G bottle and top	Fix on site and store in dark	8 Hrs.
Phosphorus Orthophosphate, Dissolved	P,G	Filter on site Cool, 4°C	48 Hrs. <i>14 days</i>
Hydrolyzable	P,G	Cool, 4°C H ₂ SO ₄ to pH<2	28 Days
Total	P,G	Cool, 4°C H ₂ SO ₄ to pH<2	28 Days

Table 3.1 (continued)

Phosphorus (cont.) Total, Dissolved	P, G	Filter on site Cool, 4°C H ₂ SO ₄ to pH<2	24 Hrs.
Silica	P only	Cool, 4°C	28 Days
Sulfate	P, G	Cool, 4°C	28 Days
Sulfide	P, G	Cool, 4°C add 2ml zinc acetate plus NaOH to pH>9	7 Days
Sulfite	P, G	None Req.	None ^f
Total Carbon	P, G	Cool, 4°C H ₂ SO ₄ or HCl to pH <2	28 Days
Total Inorganic Carbon	P, G	Cool, 4°C H ₂ SO ₄ or HCl to pH <2	28 Days
<u>Organics</u>			
BOD	P, G	Cool, 4°C	48 Hrs.
COD	P, G	Cool, 4°C	28 Days
Halogenated Hydrocarbons	G only	Cool, 4°C	7 Days
Oil & Grease	G only	Cool, 4°C H ₂ SO ₄ to pH<2	28 Days
Organic Acids	G only	Cool, 4°C	14 Days
Organic Alcohols	G only	Cool, 4°C	14 Days
Organic Carbon			
total	P, G	Cool, 4°C H ₂ SO ₄ or HCl to pH<2	28 Days
purgeable	G only	Cool, 4°C zero head space	7 Days
Pesticides	G only	Cool, 4°C	7 Days

Table 3.1 (continued)

Phenolics	G only	Cool, 4°C H ₂ SO ₄ to pH<2	28 Days
Surfactants			
MBAS	P,G	Cool, 4°C	28 Days
MTA	P,G	Cool, 4°C	24 Hrs.
Toxic Organics			
BNA	G only	Cool, 4°C	7 Days
VOA	G only	Cool, 4°C Zero head space	14 Days
NVO	G only	Cool, 4°C	7 Days
Urea	G only	Cool, 4°C	7 Days
Microbiology			
Heterotrophic Plate Count	P sterile	Cool, 4°C Na ₂ S ₂ O ₃	12 hrs ^J
Non-Saprophytic Plate Count	P sterile	Cool, 4°C Na ₂ S ₂ O ₃	4 hrs
Fecal Coliforms	P sterile	Cool, 4°C Na ₂ S ₂ O ₃	12 hrs ^J
Yeast and Molds	P sterile	Cool, 4°C Na ₂ S ₂ O ₃	12 hrs ^J
Total Count	P sterile	Cool, 4°C <i>5% glutathione</i> 10% w/v formalin	48 hrs
Gram Positives	P sterile	Cool, 4°C Na ₂ S ₂ O ₃	12 hrs ^J
Gram Negatives	P sterile	Cool, 4°C Na ₂ S ₂ O ₃	12 hrs ^J
Anaerobes	P sterile	Cool, 4°C Cysteine Na ₂ S ₂ O ₃	2 hrs
Enterics	P sterile	Cool, 4°C Na ₂ S ₂ O ₃	12 hrs ^J

Legionella

Table 3.1 (continued)

- a More specific instructions for preservation and sampling are found with each procedure as detailed in the EPA manual of Methods for Chemical Analysis of Water and Wastes. A general discussion on sampling water and industrial wastewater may be found in ASTM, Part 31, p. 72-82 (1976) Method D-3370.
- b Plastic (P) or Glass (G). For metals, polyethylene with a polypropylene cap (no liner) is preferred.
- c Sample preservation should be performed immediately upon sample collection. For composite samples each aliquot should be preserved at the time of collection. When use of an automated sampler makes it impossible to preserve each aliquot, then samples may be preserved by maintaining at 4°C until compositing and sample splitting is completed.
- d When any sample is to be shipped by common carrier or sent through the United States Mails, it must comply with the Department of Transportation Hazardous Materials Regulations (49CFR Part 172). The person offering such material for transportation is responsible for ensuring such compliance.
- e Samples should be analyzed as soon as possible after collection. The times listed are the maximum times that samples may be held before analysis and still considered valid. Samples may be held for longer periods only if the permittee, or monitoring laboratory, has data on file to show that the specific types of sample under study are stable for the longer time, and has received a variance from the Regional Administrator. Some samples may not be stable for the maximum time period given in the table. A permittee, or monitoring laboratory, is obligated to hold the sample for a shorter time if knowledge exists to show this is necessary to maintain sample stability.
- f There is no acceptable holding time established. Samples should be analyzed immediately upon collection.
- g Samples should be filtered immediately on-site before the addition of preservative for dissolved metals.
- h Should only be used in the presence of residual chlorine.
- i For samples from non-chlorinated drinking water supplies conc. H_2SO_4 should be added to lower sample pH to less than 2. The sample should be analyzed before 14 days.
- j Although eight (8) hours is given as a recommended holding time all samples should be processed as soon as practical. Microbiological populations and recovery are altered by increased storage times. Samples should not be analyzed if more than 24 hours elapse between collection and analysis.

Source of Table: From EPA manual of Methods for Chemical Analysis of Water and Waste.

authorize sample disposal by way of written notification within ten (10) days.

3.2 Analytical Methods

3.2.1 Procedures

All methods used by the participating laboratories must be approved by the ACC, the Principal Investigator, MSFC Materials and Processing Laboratory and Medical Monitor prior to use. The primary methods used are those outlined and approved by the Environmental Protection Agency (EPA) and the American Public Health Association (APHA). Secondary methods, when required, will be from recognized sources such as the Association of Official Analytical Chemists (AOAC) and the American Society for Testing Materials (ASTM). In some cases, recommended procedures or instrumental parameters for certain analyses are contained within a manufacturer's operating manual. These should only be used as guidelines when different from recommended or primary procedures. All modifications to any method as specified above must have prior written approval by the ACC, Principal Investigator, MSFC Materials and Processing Laboratory and Medical Monitor before use by a participating laboratory.

Efforts will be made to coordinate and standardize methodology between cooperating laboratories in order to eliminate methodology as a variable when comparing results and assessing validity and accuracy. Table 3.2 lists currently approved methods for analysis. Methodology is to be thoroughly documented including precision and accuracy parameters for each analysis procedure within each participating laboratory.

3.2.2 Analyst Proficiency

Analysts who perform a particular method must demonstrate proficiency in and use of any instrumentation required. Refer to Section 3.4.2.2, Instrument Qualification and Training.

3.2.3 Detection Limits

Each laboratory must demonstrate the capability of meeting the required detection limits of each method as specified in Table 3.2.

3.2.4 Methods Development

From time to time it may be necessary to develop new methods for analyzing certain parameters. In addition, modification of existing methods may be necessary to improve recovery, account for matrix effects or availability of instrumentation within a laboratory. For any new method or modification of an existing method, certain information must be obtained. First, side by side comparison with the existing method (if applicable) will be conducted for a minimum of twenty individual samples. Second, validity, accuracy and precision data must accompany the comparative results and will include, as a minimum, twenty individual measurements (Section 3.4.3.2). Third, complete written documentation of the procedure including steps outlining each aspect of the procedure as well as instrument settings and calibration data will be included. The complete documentation package is then submitted to the ACC. The ACC will verify the contents and forward copies of the data

package to the Materials and Processing Laboratory (NASA/MSFC) and Biomedical Laboratories Branch (NASA/JSC). In addition, a copy of the method may be sent to an outside laboratory for verification. Written recommendations concerning the applicability of the method will be made to the Principal Investigator. A final determination will be made by the Principal Investigator, MSFC Materials and Processing Laboratory and Medical Monitor. If approved, the method will be added to the approved list. Copies of the approved method will then be sent to the participating laboratories for incorporation into the analytical program as applicable.

The following criteria^{1,6} will be used to determine specific methods selected:

- (1) The method will measure the desired constituents in the presence of normal interferences with required precision and accuracy.
- (2) Each selected procedure will utilize available equipment and skills typically found in the laboratory.
- (3) Each selected method will have been tested for established validity.
- (4) The methods selected will be sufficiently rapid to allow for routine use for a large number of sample analyses.
- (5) The methods selected will be capable of achieving the required detection limits.

TABLE 3.2. APPROVED PHYSICAL AND CHEMICAL ANALYTICAL METHODS

PARAMETER	METHOD	TECHNIQUE	UNITS	DET'N LIMIT ^a
PHYSICAL				
conductivity	EPA 120.1	specific conductance	umho/cm	1.0
color	SM 204 A	spectrophotometric	units	1.0
dissolved gas	TBD	tbd	tbd	tbd
odor	EPA 140.1	threshold odor	TOM	15
particulates	tbd	Climet CI-1000	tbd	tbd
pH	EPA 150.1	electrometric	pH units	0-14
total solids	EPA 160.3	gravimetric	mg/l	10
total dissolved solids	EPA 160.2	gravimetric	mg/l	10
total suspended solids	EPA 160.1	gravimetric	mg/l	10
turbidity	EPA 180.1	nephelometric	NTU	0
INORGANIC NONMETALS				
alkalinity	EPA 310.1	titrimetric	mg/l as CaCO ₃	1.0
ammonia	EPA 300.7	ion chromatography	mg/l	0.05
bromide	SM 429	ion chromatography	mg/l	0.05
chloride	SM 429	ion chromatography	mg/l	0.04
chlorine, total residual	EPA 330.5	colorimetric	mg/l	0.1
fluoride	SM 429	ion chromatography	mg/l	0.02
iodide	SM 414 A	leuco crystal violet	mg/l	0.1
iodine, total	SM 414 A	leuco crystal violet	mg/l	0.1
nitrate	SM 429	ion chromatography	mg/l	0.1
nitrogen, total	SM 420	colorimetric	mg/l	1.0
phosphate	SM 429	ion chromatography	mg/l	0.3
sulfate	SM 429	ion chromatography	mg/l	0.15
sulfide	AU 107	ion chromatography	mg/l	0.05
total carbon	SM 505 B	UV persulfate/NDIR	mg/l	0.2
total inorganic carbon	SM 505 B	UV persulfate/NDIR	mg/l	0.2
INORGANIC METALS				
arsenic	EPA 206.2	AAG	mg/l	0.002
barium	EPA 200.7	ICP	mg/l	0.002
	EPA 208.2 ^b	AAG	mg/l	0.002
cadmium	EPA 200.7	ICP	mg/l	0.01
calcium	EPA 200.7	ICP	mg/l	0.01
chromium	EPA 200.7	ICP	mg/l	0.01
	EPA 218.2 ^b	AAG	mg/l	0.01
copper	EPA 200.7	ICP	mg/l	0.01
	EPA 220.2 ^b	AAG	mg/l	0.01
iron	EPA 200.7	ICP	mg/l	0.02
	EPA 236.2 ^b	AAG	mg/l	0.02
lead	EPA 239.2	AAG	mg/l	0.001
magnesium	EPA 200.7	ICP	mg/l	0.03
	EPA 242.1 ^b	AAF	mg/l	0.03
manganese	EPA 200.7	ICP	mg/l	0.005
	EPA 243.2 ^b	AAG	mg/l	0.005
mercury	EPA 245.1	Hg analyzer	mg/l	0.0002

TABLE 3.2. (continued)

PARAMETER	METHOD	TECHNIQUE	UNITS	DET'N LIMIT ^a
INORGANIC METALS (CONT.)				
nickel	EPA 200.7	ICP	mg/l	0.03
potassium	EPA 300.0	ion chromatography	mg/l	0.05
selenium	EPA 270.2	AAG	mg/l	0.002
silver	EPA 200.7	ICP	mg/l	0.03
sodium	EPA 300.0	ion chromatography	mg/l	0.02
zinc	EPA 200.7	ICP	mg/l	0.01
	EPA 289.2 ^b	AAG	mg/l	0.01
ORGANICS				
total organic carbon	SM 505 B	UV persulfate/NDIR	ug/l	200
specific organics:				
acid extractables	EPA 625/525 ^c	GC/MS	ug/l	see note d
base/neutral ext.	EPA 625/525 ^c	GC/MS	ug/l	see note d
volatiles	EPA 624/524 ^c	GC/MS	ug/l	see note d
nonvolatiles	tbd	tbd	ug/l	see note d
phenols	EPA 625/525 ^c	GC/MS	ug/l	see note d
cyanide	AU 107	ion chromatography	ug/l	20
halogenated hydrocarbons	EPA 625/525 ^c	GC/MS	ug/l	see note d
organic acids	TBD	GC	ug/l	see note d
organic alcohols	TBD	GC/FID Head space	ug/l	0.2
pesticides	EPA 608/508 ^c	GC/ECD	ug/l	see note d
MISCELLANEOUS				
urea	TBD	ion chromatography	ug/L TBD	100 ppb tbd
methylene blue active substances (MBAS)	EPA 425.1	colorimetric	ug/l	100

^aThe detection limits listed represent either the limit or the applicable range stated in the respective method. For those methods for which detection limits or applicable ranges are not defined, the limits listed in this table reflect the minimum detection limit anticipated to be required to meet phase III objectives.

^bSecondary method

^cFor future tests, series 500 methods for drinking water may be required.

^dSpecies-specific

3.3 Interlaboratory Control

The interlaboratory control program is a systematic testing program in which uniform samples are analyzed by all participating laboratories to assess the continuing capability, relative performance and improvement in identified weak areas of each. The purposes of interlaboratory testing are as follows:

- o To provide a measure of the precision and accuracy of analytical methods run routinely by participating laboratories
- o To estimate the accuracy and precision of results between laboratories
- o To identify weak methodology
- o To identify inadequate equipment and instrumentation
- o To detect training needs
- o To upgrade the overall quality of the laboratory performance

3.3.1 Analytical Control Samples

Analytical control samples will consist of initial qualification samples, monthly verification samples, blind duplicate samples and random blind verification samples. All analytical control samples, except for the blind duplicates, will be purchased by the ACC and certified by the vendor for authenticity. Efforts will be made to mimic individual matrices and analysis parameters where applicable.

3.3.2 Initial Qualification

Initially each participating laboratory will be sent a series of three (3) qualification check samples. Each participating laboratory will be identified by a laboratory code

in order that performance results remain anonymous to other participants. Qualification samples will be certified purchased samples and will include a wide range of analytes in order that performance can be determined for individual classes of compounds. Tentative parameters and ranges of analyte concentrations are presented in Table 3.3. Results of the completed analyses will be reported directly to the ACC within two weeks of receipt. Performance results of each laboratory will in turn be reported to the Principal Investigator and the MSFC Materials and Processing Laboratory representative. The ACC will report the results of the qualification samples directly to the individual laboratories. If an area is identified as weak for a participating laboratory, a plan to correct these deficiencies must be submitted to the ACC prior to continued participation by that laboratory. Subsequent results provided in an identified deficient area will be labeled as "suspect" until the deficiencies are corrected. Improvement will be monitored through the qualification program. If an area deficiency is not corrected by the third qualification sample, the laboratory will be restricted to only those analyses for which it can demonstrate proficiency or excluded from further participation entirely. The decision to restrict or eliminate a laboratory from participation will be made by the Principal Investigator, MSFC Materials and Processing Laboratory and/or Medical Monitor.

**TABLE 3.3 Typical Parameters and Concentrations for Qualification,
Verification and Reference Check Samples**

Parameter	Concentration Range
PHYSICAL	
conductivity	500-2500 umho/cm
pH	6-10 pH units
Total solids	500-2000 mg/l
total dissolved solids	500-2000 mg/l
total suspended solids	10-120 mg/l
turbidity	0.3-10 NTU
INORGANIC NONMETALS	
alkalinity	100-300 mg/l as CaCO ₃
ammonia	1-20 mg/l
chloride	50-400 mg/l
chlorine, total residual	0.5-3 mg/l
fluoride	1-20 mg/l
nitrate	1-20 mg/l
nitrogen, total	1-20 mg/l
phosphate	1-10 mg/l
sulfate	50-400 mg/l
INORGANIC METALS	
arsenic	10-250 ug/l
barium	20-2000 ug/l
cadmium	5-500 ug/l
calcium	10-200 mg/l
chromium	20-2000 ug/l
copper	20-2000 ug/l
iron	20-2000 ug/l
lead	20-2000 ug/l
magnesium	5-200 mg/l
manganese	20-2000 ug/l
mercury	1-20 ug/l
nickel	20-2000 ug/l
potassium	10-300 mg/l
selenium	5-500 ug/l
silver	5-500 ug/l
sodium	10-300 mg/l
zinc	50-1000 ug/l

TABLE 3.3 (continued)

Parameter	Concentration Range	
ORGANICS		
total organic carbon	10-100 mg/l	
specific organics:		
acid extractables	10-250 ug/l	see note a
base/neutral ext.	10-250 ug/l	see note b
volatiles	1-200 ug/l	see note c
phenols	25-500 ug/l	
cyanide	25-500 ug/l	
halogenated hydrocarbons	0.01-10 ug/l	see note d
halomethanes	1-100 ug/l	see note e
pesticides	0.01-10 ug/l	see note f

Note a includes 3-6 acid extractable compounds each within the listed concentration range

Note b includes 6-12 base/neutral compounds each within the listed concentration range

Note c includes 2-10 volatile compounds each within the listed concentration range

Note d includes 1-2 common Aroclors each within the listed concentration range

Note e includes up to five halomethanes each within the listed concentration range

Note f includes 3-8 pesticides each within the listed concentration range

3.3.3 Verification Samples

Verification samples will be provided to the participating laboratories on a monthly basis. The purpose of the verification samples will be to monitor the continual performance of the participating laboratories and to provide a mechanism for monitoring improvement of any identified deficient area(s) within a participating laboratory. The samples will consist of certified purchased samples containing a wide variety of analytes as identified previously (Table 3.3).

3.3.4 Blind Samples

Blind samples are those samples which are prepared by someone other than the analyst or laboratory performing the work. With the exception of the duplicate samples, all blind samples submitted to the participating laboratories will be certified for authenticity by the vendor. The blind samples will be used as a independent check for accuracy and precision.

3.3.4.1 Duplicate Samples

At random time intervals, blind duplicate samples will be collected or prepared and submitted to each laboratory. Blind duplicate samples may be either process samples collected and submitted as two separate samples within a single batch or reference samples which are split and submitted as two separate samples within a given batch. These samples will be used by the ACC to monitor precision of the overall laboratory procedure.

3.3.4.2 Split Samples

At random time intervals, samples collected will be split and submitted to two or more participating laboratories. These samples will be used to determine the precision between the individual laboratories for specific method parameters.

3.3.4.3 Reference Samples

At random time intervals, blind reference samples will be submitted to the individual participating laboratories. Reference samples may be submitted in duplicate or singly. These samples will be used by the ACC as a check for precision and/or accuracy for individual parameters. The parameters and concentration ranges of the blind reference samples will be as previously identified (Table 3.3).

3.3.5 Reference Laboratory

The use of a single laboratory to establish the authenticity of a test sample is problematic at best. However, reference laboratories may be identified based on experience, recognition and demonstrated performance in specific areas. Samples chosen at random as well as any samples labeled as "key samples" will be sent to reference laboratories for verification. Random samples for verification will be chosen by the ACC. "Key samples" may be identified to the ACC by either the Principal Investigator or the Medical Monitor.

3.3.6 Laboratory Audits

Both formal and informal laboratory audits will be conducted during the course of this study. Informal audits may consist of personal or telephone interviews with individual laboratory personnel or the Directors/Managers of the participating laboratories. Informal audits will not be announced and will generally be used to discuss specific problems, individual results submitted or methods used by the laboratory. The informal audits will be conducted by the ACC.

Formal audits will consist of both off-site and on-site inspection activities. Formal audits will be announced and coordinated with the Director/Manager of the participating laboratory. Results and suggestions resulting from all formal audits will be provided to the individual Laboratory Director/Manager in writing. Formal audits will be conducted by two or more individuals representing the ACC, the Principal Investigator, Medical Monitor and the MSFC Materials and Processing Laboratory.

3.3.6.1 Off-Site Inspections

Off-site inspections will consist of review of laboratory documents, raw data, quality assurance data, protocols, sampling plans and any other material deemed pertinent by either the Principal Investigator, Medical Monitor, MSFC Materials and Processing Laboratory or ACC. The review may take place with or without laboratory personnel involvement. Off-site inspections will be conducted as required.

3.3.6.2 On-Site Inspections

On-site inspections will be conducted from time to time by an audit team. Notification will be given to the Laboratory Director/Manager the week before the inspection is to take place. On-site inspections will be conducted at random or if a significant problem is suspected within the laboratory organization. Generally, routine audits will be conducted for each participating laboratory initially and then twice per year. An on-site inspection will be conducted within a single working day and will be structured such that it will have minimal effect on the normal laboratory operation. During the inspection, the audit team will interview laboratory personnel, management personnel and the Laboratory Control Coordinator. They will also inspect recordkeeping procedures, sample handling and control procedures, analysis procedures, laboratory safety, instrumentation, staff qualifications and experience, workload and understanding of analysis procedures. Upon conclusion, an oral debriefing will be made to the Laboratory Management outlining the findings. A final written report will be prepared outlining problems noted and subsequent recommendations.

3.3.7 Laboratory Certification

With the exception of the participating clinical laboratories, laboratory certification or accreditation is not a requirement for participation at this time. However, each participating laboratory is strongly encouraged to pursue certification independently. Certification or accreditation programs help to improve and maintain the validity, accuracy and

precision of test data and promote the acceptance of test data by the users. In addition, test data produced by certified or accredited laboratories are more readily accepted by other laboratories without further testing. It should be noted that laboratory certification for participation in recipient mode testing or analysis of samples relating to donor safeguards will undoubtedly be a future requirement.

3.4 Intralaboratory Control

Intralaboratory control describes those procedures used within a given laboratory to produce and maintain quality results. An adequate intralaboratory control program provides a continuing measurement of performance of individual analysts as well as instrumentation.

3.4.1 Laboratory Documentation

All operations, procedures, methodologies and reporting pertinent to the laboratory should be thoroughly documented for both management and laboratory personnel.

3.4.1.1 Standard Operating Procedures Document

Each participating laboratory will have a written Standard Operating Procedures (SOP) document. As a minimum the laboratory SOP document should address the following:

- o Personnel
- o Organization and Management
- o Special Situations and Emergencies (who to contact)
- o Facilities and Services
- o Instrumentation
- o General Procedures
- o Analytical Methods
- o Log of personnel who have read the document

3.4.1.2 Analytical Control Document

Each participating laboratory will have a written Analytical Control Document. As a minimum the AC document should address the following:

- o Sample Collection and Handling
- o Sample Tracking
- o Quality Control Procedures
- o Quality Assurance Procedures
- o Data Handling and Reporting
- o Log of personnel who have read this document

3.4.2 Personnel

3.4.2.1 Skill Level

The skill level of personnel and understanding of relevant procedures and instrumentation are essential to providing quality results in an analytical laboratory. Lead chemists should possess an advanced degree and/or equivalent experience. Staff chemists should possess a degree in chemistry. Ideally, the program of study should be accredited by the American Chemical Society (ACS). Both lead and staff chemists should maintain a professional status as a chemist. Laboratory analysts in the chemistry laboratory should minimally possess a high school diploma and have received thirty days on the job training which should include specific instruction on the procedure or instrumentation he/she is expected to operate. Minimal guidelines have been established in order to relate necessary skill level requirements to the complexity of the analytical and instrumental procedures involved¹. These guidelines are presented in Table 3.4. All laboratory personnel should be supervised by an experienced professional scientist.

TABLE 3.4 SKILL RATING FOR STANDARD ANALYTICAL OPERATIONS¹

Measurement	Skill Required
Simple Instrumentation	
pH	1
Conductivity	1
Turbidity	1
Color	1
Dissolved Oxygen	1,2
Simple Volumetric	
Alkalinity	1
Acidity	1
Chloride	1
Hardness	1
Simple Gravimetric	
Solids	1,2
Simple Colorimetric	
Nitrate	2
Nitrite	2
Sulfate	2
Silica	2
Complex, Volumetric or Colorimetric	
BOD	2,3
COD	2,3
TKN	2,3
Ammonia	2,3
Oil and Grease	2,3
Fluoride	2,3
Cyanide	2,3
Special Instrumentation and Procedures	
TOC	2,3
Inorganic non-metals (Ion Chromatography)	2,3
Metals (Atomic Absorption)	2,3
Metals (Inductively Coupled Plasma)	2,3
Organics (Gas Chromatography)	3,4
Organics (Liquid Chromatography)	3,4
Methods Development	3,4

1 semi-skilled sub-professional; comparable to GS-3 through GS-5.

2 experienced aide (sub-professional) or a professional with modest training and experience; comparable to GS-4 through GS-7.

3 professional with good background and experience in analytical techniques; comparable to GS-7 through GS-11.

4 professional with specialization, requires interpretation of results; comparable to GS-9 through GS-13.

3.4.2.2 Instrument Qualification and Training

It is generally recognized that for experienced, higher grade personnel, formal training in special fields, techniques and instrumentation should be a mandatory requirement. Similar formal training for lower grade personnel is not readily available. For these individuals an in-house training program should be established to improve analytical capabilities, conceptual understanding, instrumental procedures and quality performance. This can be accomplished through an established program of working with more experienced analysts, cross training, laboratory seminars and exposure to pertinent literature.

Notification of any changes to the laboratory staff within a participating laboratory must be made directly to the ACC. The ACC will then notify the Principal Investigator, MSFC Materials and Processing Laboratory's representative and Medical Monitor of these changes.

3.4.2.3 Laboratory Control Coordinator

Each participating laboratory is expected to designate a single person separate from the analysis personnel whose sole responsibility is surveillance and monitoring of in-house analytical control activities.

3.4.3 Analytical Controls

The terms Quality Control (QC) and Quality Assurance (QA) refer to the whole spectrum of laboratory practices designed to monitor and assure accuracy, precision, and validity of the

results measured. In analytical laboratories which are not involved in industrial processes, the following QA and QC definitions are generally accepted. Quality control refers to special procedures for demonstrating the validity of results. The primary objective of quality control is to provide a system of activities to assure the quality of the analyses. Quality assurance refers to all elements of proper technique which minimize errors. Therefore, the goal of quality assurance is to provide a system of activities to assure that the quality control system is performing properly by meeting the program requirements for reliability.

The QA/QC program will encompass and support all laboratory operations which include sample collection (if performed by the laboratory), sample tracking, analytical methods, instrumentation, policies, and documentation/reporting activities. It is essential that standard written practices be established within the laboratory to promote efficient and effective operation and to assure that QA/QC program procedures are incorporated into the operational structure. Analytical control must begin with sample collection and must not end until the resultant data have been reported. Conscientious use and understanding of analytical control measures among field personnel, analytical personnel, and management personnel is imperative.

Because of the importance of laboratory analyses in determining practical courses of action that may be followed, quality assurance programs to insure the reliability of the water and wastewater data are essential.

The QA/QC program should facilitate the following specific objectives:

- (1) Define the responsibilities of laboratory personnel associated with a given project.
- (2) Associate reliability estimates for the level of quality associated with any analytical method, system, or process.
- (3) Assist in the early recognition of deficiencies or problems which might affect data quality.
- (4) Enable the Laboratory Control Coordinator to take corrective action as required to insure the validity of laboratory data.
- (5) Enhance the utility of all data by requiring adequate documentation to support anticipated decision-making.
- (6) Provide an organizational plan which may be used to implement quality control in laboratory operations.

3.4.3.1 Intralaboratory Control Samples

With each batch of analyses the following samples will be included as a minimum:

Prior To Analysis Of Unknowns

- 1 water, reagent or solvent blank, as applicable
- 1 preservative blank, if applicable
- 1 method blank
- 1 reference sample
- 3-5 standards

Interspersed With Unknown Samples

- 1 spiked sample for every 20 unknowns or per analysis batch
- 1 set of duplicate samples for every 20 unknowns or per analysis batch
- 1 standard for every 20 unknowns

Following Analyses of Unknowns

- 1 reference sample

Each of these samples are described in the following Sections.

3.4.3.2 DEFINITIONS

The following definitions have been suggested by the USEPA in order to standardize their use¹³.

3.4.3.2.1 Internal Standard

A pure analyte(s) added to a solution in known amount(s) and used to measure the relative responses of other method analytes and surrogates that are components of the same solution. The internal standard must be an analyte that is not a sample component.

3.4.3.2.2 Surrogate Analyte

A pure analyte(s), which is extremely unlikely to be found in any sample, and which is added to a sample aliquot in known amount(s) before extraction and is measured with the same procedures used to measure other sample components. The purpose of a surrogate analyte is to monitor method performance with each sample.

3.4.3.2.3 Laboratory Duplicates (LD1 and LD2)

Two sample aliquots taken in the analytical laboratory and analyzed separately with identical procedures. Analyses of LD1 and LD2 give a measure of the precision associated with laboratory procedures, but not with sample collection, preservation, or storage procedures.

3.4.3.2.4 Field Duplicates (FD1 and FD2)

Two separate samples collected at the same time and place under identical circumstances and treated exactly the same throughout field and laboratory procedures. Analyses of FD1 and FD2 give a measure of the precision associated with sample collection, preservation and storage, as well as with laboratory

procedures.

3.4.3.2.5 Laboratory Reagent Blank (LRB)

An aliquot of reagent water that is treated exactly as a sample including exposure to all glassware, equipment, solvents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.

3.4.3.2.6 Field Reagent Blank (FRB)

Reagent water placed in a sample container in the laboratory and treated as a sample in all respects, including exposure to sampling site conditions, storage, preservation and all analytical procedures. The purpose of the FRB is to determine if method analytes or other interferences are present in the field environment.

3.4.3.2.7 Laboratory Performance Check Solution (LPC)

A solution of one or more compounds used to evaluate the performance of the instrument system with respect to a defined set of method criteria.

3.4.3.2.8 Laboratory Fortified Blank (LFB)

An aliquot of reagent water to which known quantities of the method analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether the methodology is in control, and whether the laboratory is capable of making accurate and precise measurements at the required method detection limit.

3.4.3.2.9 Laboratory Fortified Sample Matrix (LFM)

An aliquot of an environmental sample to which known quantities of the method analytes are added in the laboratory. The LFM is analyzed exactly like a sample, and its purpose is to determine whether the sample matrix contributes bias to the analytical results. The background concentrations of the analytes in the sample matrix must be determined in a separate aliquot and the measured values in the LFM corrected for background concentrations.

3.4.3.2.10 Stock Standard Solution

A concentrated solution containing a single certified standard that is a method analyte, or a concentrated solution of a single analyte prepared in the laboratory with an assayed reference compound. Stock standard solutions are used to prepare primary dilution standards.

3.4.3.2.11 Primary Dilution Standard Solution

A solution of several analytes prepared in the laboratory from stock standard solutions and diluted as needed to prepare calibration solutions and other needed analyte solutions.

3.4.3.2.12 Calibration Standard (CAL)

A solution prepared from the primary dilution standard solution and stock standard solutions of the internal standards and surrogate analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

3.4.3.2.13 Quality Control Sample (QCS)

A sample matrix containing method analytes or a solution of method analytes in a water miscible solvent which is used to fortify reagent water or environmental samples. The QCS is

obtained from a source external to the laboratory, and is used to check laboratory performance with externally prepared test materials.

3.4.3.3 Control Charts

Quality control charts will be developed to evaluate validity, accuracy and precision. Inherent in this approach is the recognition that variations exist in every method. Where variations do not seem to exist, either the device used for measuring the process is not sensitive enough, or the analyst is not performing a procedure properly.

The emphasis for conducting accuracy and precision checks and maintaining quality control charts is to ensure and document the continuing validity of laboratory data adequately. Therefore, it is imperative that analytical control measures be applied daily to provide constant quality control monitoring.

Valid accuracy and precision data must be developed for each analyst using a particular method and specific instrumentation. Poor accuracy data may result from analytical errors such as inaccurate dilution techniques, incorrect volume/weight measurements, or improper calibration of the equipment. Low precision is likely to result from low instrument sensitivity or other factors beyond the analyst's control. Therefore, a situation may exist where accuracy is high, but precision is low, or vice versa precision may be high and accuracy low. From the time a sample is logged into the laboratory until the data is reported, the analyst has the responsibility to perform all work in a skillful manner to assure high precision and accuracy within

the scope of his/her control for all sample handling and analysis procedures.

3.4.3.3.1 Validity

To insure that valid data is continually produced, systematic daily checks must show that the test results are reproducible, and that the methodology is actually measuring the quantity of the specified constituent in each sample. Validity checks are performed by analysis of a known reference sample for each parameter immediately preceding the analysis of unknown samples. Validity checks cannot be performed for methods for which no standard solution can be obtained. These methods include certain physical characteristics and microbiological procedures.

The accepted concentration value and upper and lower control limits are plotted over time. Acceptable control limits are defined as the accepted concentration value plus and minus three standard deviations, where fifty percent or greater of the determinations fall within one standard deviation.

A reference sample will be run for each parameter prior to analysis of unknown samples and concentration values plotted on the control chart. The laboratory name, analysis number, parameter, method, sample concentration range, analyst's name, and date will be identified on the control chart. Control charts will be reviewed and maintained by the Laboratory Control Coordinator.

If analysis of reference samples reveals an out-of-control situation, i.e. a value falls outside the control limits, the

problem will be identified and resolved prior to analysis of unknown samples.

3.4.3.3.2 Accuracy

Accuracy refers to the agreement between the amount of a constituent measured by a particular method and the actual amount of the constituent present in the sample. There are two categories of samples employed for evaluating accuracy. The first of these entails the use of reference samples previously described in Section 3.4.3.3.1. The second category incorporates the use of spiked samples in which a known quantity of a reference standard containing a particular constituent is added to a test sample.

Accuracy cannot be determined for certain methods where reference standards are not available, and therefore cannot be added in known quantities to test samples for percent recovery determinations.

Accuracy control charts are established to utilize the data obtained from reference and spiked samples. For both the reference samples and the spiked samples, the percentage recovery is calculated and plotted.

A linear relationship exists between the percentage recovery and the known concentrations of standards and spikes⁷. The data from reference samples and spiked samples should be collected and percentage recovery calculations made for a minimum of twenty analyses in order to establish control chart parameters. The control chart is not valid if less than fifty percent of the initializing data fall within the accepted value plus and minus

one standard deviation. In addition, none of the initializing data may fall outside the upper and lower control limits¹. Accuracy control charts will be identified by the laboratory name, parameter, method, sample concentration range, analyst, and date. Control charts will be reviewed and maintained by the Laboratory Control Coordinator.

3.4.3.3.3 Precision

Precision refers to the reproducibility of a method when it is repeated in a homogenous sample, regardless of whether or not the observed concentrations are representative of the true concentrations. Since precision is dependent upon concentration, control charts will be developed or applied within limited concentration ranges.

Duplicate samples are prepared and analyzed in order to evaluate precision, excluding results below the detection limit, and including analyses of the same reference sample performed on the same day. Precision can be monitored for individual analysts or between analysts. Precision can be measured for all methods. The control chart is not valid if less than fifty percent of the initializing data fall within the accepted value plus or minus one standard deviation. In addition, none of the initializing data may fall outside the upper and lower control limits of three standard deviations¹.

Precision control charts will be identified by the laboratory name, parameter, method, concentration range, analyst, and date. Control charts will be reviewed and maintained by the Laboratory Control Coordinator.

4.0 REQUIREMENTS FOR MICROBIOLOGICAL ANALYSIS

Microbiological parameters measure living organisms which continually change over time and in response to environmental conditions. Because absolute values do not exist for microbiological parameters, spiked samples cannot be prepared for routine evaluations of accuracy within the microbiology laboratory. Therefore, a Laboratory Control Program for microbiological analysis must address the control of laboratory operations, analytical procedures and analyst precision. Subsequently, control procedures governing sample collection and handling, personnel, facilities, methodology, supplies and equipment must be continuously monitored¹. In addition, validity checks, positive and negative controls, sterility checks, replicate analysis and the use of verification/confirmation procedures must be used to provide analytical controls. As a minimum the following will be addressed in the intralaboratory control program for the microbiology laboratory:

4.1 General Operations

4.1.1 Laboratory Organization and Management

The microbiology laboratory's organization and management must be clearly defined. Requirements and responsibilities for each management and staff position will be documented. The laboratory will maintain a current SOP document specific to the microbiology laboratory describing approved procedures and techniques to be followed by all personnel.

As a minimum, the SOP document for the microbiology laboratory should address the following:

- o Facilities and Personnel
- o Sample Collection and Handling
- o Laboratory Equipment and Instrumentation
- o Laboratory Supplies
- o Media and Reagent Preparation
- o Reference Cultures
- o General Procedures
- o Analytical Methods
- o Analytical Control Procedures
- o Log of personnel who have read the document

4.1.2 Laboratory Personnel

The microbiology laboratory should have similar personnel and professional levels as found in a college microbiology program. Lead microbiologists should possess an advanced degree and/or equivalent experience and maintain a professional status as a microbiologist. Staff microbiologists should possess a degree from an accredited institution and have met certain course requirements⁸. These are given in Figure 3-4. In addition, staff microbiologists should have a minimum of one year bench experience in sanitary (water, milk or food) microbiology and received a minimum of two weeks supplemental training from a federal agency, state agency or university for each laboratory procedure he/she is expected to perform⁵. Ideally, staff microbiologists should be certified by the American Academy of Microbiology (AAM) or the American Society of Clinical Pathologists (ASCP). Laboratory analysts in the microbiology laboratory should minimally possess a high school diploma and have received 30 days on the job training including specific instruction in aseptic technique and safety considerations relating to biological hazards. In addition, the

FIGURE 3.4 MINIMUM ELIGIBILITY REQUIREMENTS FOR MICROBIOLOGISTS

Certification Level	Experience	Degree ²	Microbiology & Biological Sciences	Chemistry	Physics	Mathematics
A. Conditional Registrant						
	not required before examination	BS/BA awarded by selected exam date	20 semester hours or 30 quarter hours in microbiology plus 10 semester hours or 15 quarter hours in other biological sciences	16 semester hours or 24 quarter hours	3 semester hours or 5 quarter hours	through college algebra or equivalent
B. Registered Microbiologist RN(AAM)						
	1 Year laboratory (within the last 3 years)	BS/BA awarded by selected exam date	20 semester hours or 30 quarter hours in microbiology plus 10 semester hours or 15 quarter hours in other biological sciences	16 semester hours or 24 quarter hours	3 semester hours or 5 quarter hours	through college algebra or equivalent
C. Specialist Microbiologist SH(AAM)						
	4 years post-baccalaureate laboratory (within the last 7 years)	MS/MA or Doctorate	30 semester hours or 45 quarter hours in microbiology	16 semester hours or 24 quarter hours	3 semester hours or 5 quarter hours	through college algebra or equivalent
Alternate Pathway						
	7 years post-baccalaureate laboratory (within the last 10 years)	BS/BA	20 semester hours or 30 quarter hours in microbiology plus 10 semester hours or 15 quarter hours in other biological sciences	16 semester hours or 24 quarter hours	3 semester hours or 5 quarter hours	through college algebra or equivalent

1. If the applicant can demonstrate to the satisfaction of the Certification Board through application and reference forms that he or she meets the eligibility requirements, the candidate will be notified of admission to examination.
2. Degree must be awarded by a college of university accredited by one of the United States' regional accrediting agencies. Microbiology is understood to involve the study of microorganisms and may include: a) courses in bacteriology, immunology, mycology, parasitology, rickettsiology, tissue culture and virology; b) food, dairy, diagnostic, industrial, sanitation,

analyst should have received one week supplemental training from a federal agency, state agency or university for each procedure he/she is expected to perform⁵. Laboratory personnel should be supervised by an experienced professional scientist.

4.1.3 Laboratory Facilities

The laboratory facilities must be neat and organized into appropriate work stations. The space allotted for microbiological procedures must be adequate for the work load and number of employees assigned to this area. A minimum of six (6) linear feet of bench space for each analyst assigned to the laboratory is recommended⁵.

4.1.4 Laboratory Cleanliness

High standards for cleanliness must be maintained in microbiological work areas. This is imperative not only for protection of the worker but also for protection of samples from outside contamination. A routine program should be implemented and monitored to minimize and identify potential contamination sources of both the laboratory and samples. This program should include scheduled cleaning and disinfection, monitoring UV lamp intensity and monitoring water, surface and air contamination within the laboratory.

4.1.5 Recordkeeping

Written documentation of all laboratory practices and activities is imperative to adequate laboratory control. This may be accomplished by maintaining activity records and log books documenting samples received by the laboratory, specific

laboratory procedures and practices, verification of proper equipment operation, results of positive and negative controls, sterility checks, validity checks and replicate analyses.

4.2 Instrument Use Procedures

Instruments and equipment used in the laboratory must be kept in good working order and operated properly. This section describes the controls for maintaining and verifying that all laboratory instrumentation and equipment is in good working order.

4.2.1 Preventative Maintenance

A regular program for scheduled maintenance of instruments and equipment will be maintained by the laboratory.

4.2.2 Validation of Proper Operation

All equipment must be validated during use that it is performing satisfactorily and within control limits set by the procedure being performed or the manufacturer's specification, as applicable.

4.2.3 Calibration

All instrumentation and equipment in use within the laboratory will be calibrated on a regularly scheduled basis.

4.3 Special Equipment

The microbiology laboratory has specific and special equipment requirements. Procedures for the proper operation of this equipment should be included in the microbiology laboratory's SOP document. The minimum requirements for an adequately equipped microbiology laboratory are listed below.

Due to the diverse nature of microbiological parameters which may be monitored by a given laboratory, no recommendation on the specific type and quantity of equipment is specified herein. Specific equipment requirements are identified in the referenced methodologies.

The minimum equipment requirements for a participating laboratory to conduct specific microbiological analyses include:

- o autoclave
- o pH meter with temperature probe
- o vortex mixers
- o gram staining equipment
- o microbial identification equipment
- o analytical balance(s)
- o hot plate/stirrers
- o drying oven
- o refrigerator
- o freezer
- o incubators
- o anaerobic chambers
- o water baths
- o membrane filtration assembly
- o UV sterilization unit
- o vacuum pump w/water trap
- o laminar flow hood
- o Class II biological safety cabinet
- o water purification system
- o microscopes

4.4 Methods and Procedures

4.4.1 Sample Collection and Handling

Refer to Section 3.1.

4.4.2 Preparation of media and reagents

4.4.2.1 Water

Water quality in the microbiology laboratory is of extreme importance. Table 4.4 lists the acceptable limits of water quality for microbiological analysis and the frequency of verification.

**TABLE 4.4 Water Quality Requirements and Monitoring Frequency
For Microbiological Analyses⁵**

Analysis Parameters	Monitoring Frequency	Limit
Chemical Tests		
Conductivity	Continuously	<2 umhos/cm
pH	Each Use	5.5-7.5
TOC	Monthly	<1 mg/L
Heavy Metals (Cd, Cr, Cu, Ni, Pb & Zn)	Monthly	<0.5 mg/L
Heavy Metals (total)	Monthly	≤1 mg/L
Ammonia/Organic Nitrogen	Monthly	<0.1 mg/L
Residual Chlorine	Monthly	Not Detectable
Bacteriological Tests:		
Heterotrophic Plate Counts	Weekly	<1000 CFU/mL
Water Quality Tests	Annually	0.8-3.0 ratio
Use Test	Annually	Student's t ≤ 2.78

4.4.2.2 Reagents

Only chemicals of ACS reagent grade or equivalent may be used. Impurities may provide growth promoting or growth inhibiting effects or interfere with the desired reaction. All chemicals should be labeled with date received and date opened. Opened chemicals should be stored in a cool dry place unless otherwise noted and should not be kept for more than one year. Reagents should be prepared using Class A (or calibrated) volumetric flasks and transferred to appropriate containers for storage. Prepared reagents will be labeled with name, concentration, date prepared, preparer's initials and expiration date.

4.4.2.3 Culture Media

The isolation, recovery and maintenance of microorganisms depends upon the quality of ingredients and the proper preparation of the culture media. If available, commercially formulated media will be used. Unopened bottles of media will be stored in a cool dry place and may be kept for up to two years. Culture media should be dated when received and when opened for use. Opened bottles of media may be stored for up to one year.

Culture media should be prepared as directed by the supplier. Media should be dissolved in deionized or distilled water (see Section 4.4.2.1) using heat and continuous stirring. Bring media to a gentle boil and remove from heat immediately to avoid scorching. If required, autoclave media for the minimum time specified. The pH of the media should be checked and adjusted for every batch. Media must be allowed to cool or pH measurements must be compensated for temperature since pH is temperature dependent. Sterility checks and positive and negative control checks are made for each batch of medium. All information is recorded in the media preparation log book.

Prepared media may be stored only for a finite time⁵. Table 4.5 lists the recommended shelf life for prepared media. All prepared media will be stored in the dark at 4° C until use. Culture media must be allowed to equilibrate to room temperature before use in order to prevent temperature shock of the microorganisms. Under no circumstances may media be reautoclaved and used.

TABLE 4.5 Recommended Shelf Life for Prepared Media⁵

Media Type	Shelf Life
Membrane filter (MF) broth in screw-cap flasks at 4 °C	96 hours
MF agar in plates with tight fitting covers at 4 °C	2 weeks
Agar or broth in loose-cap tubes at 4 °C	1 week
Agar or broth in tightly closed screw-cap tubes at 4 °C	3 months
Poured agar plates with loose fitting covers in sealed plastic bags at 4 °C	2 weeks

4.4.3 Glassware

Special precautions and procedures must be followed in the cleaning and preparation of glassware for use in microbiological determinations. All glassware should be examined and chipped or badly etched glassware should be discarded. Extra care must be taken to insure that growth promoting or inhibitory compounds are removed prior to use. Several methods are available and recommended for verification of glassware before use⁵. These include:

- o visual inspection of excessive water beading
- o acid or alkaline residues
- o residual detergent
- o growth promotion/inhibition

4.4.4 Plasticware

Plasticware when used should be presterilized and disposable.

4.4.5 Analytical Methods

The approved analytical methods for the analysis of microbiological parameters are presented in Table 4.6. Substitution or modification of any listed method must have the prior written approval by the ACC, Principal Investigator, MSFC Materials and Processing Laboratory and Medical Monitor (see Section 3.2.4).

4.4.6 Specialized Procedures

From time to time specialized procedures may be required and conducted by participating laboratories. These would include microbiological identification, anaerobe isolation and enumeration, isolation and identification of pathogenic microorganisms, detection and enumeration of viruses and the detection, isolation and enumeration of biofouling organisms. For these cases, complete written documentation of the procedures to be followed is required (Section 3.2.4). In addition, the skill level of personnel required for these tasks will be those outlined for Lead microbiologists (Section 4.1.2).

4.5 Analytical Control Procedures

4.5.1 Positive and Negative Controls

Positive and negative control organisms (ATCC reference cultures) must be used in every phase of laboratory operation. This would include the preparation of culture media, determining physiological and biochemical characteristics, selective isolation and enumeration procedures and identification of isolates. The proper use of reference cultures must be described and documented.

4.5.2 Replicate Analysis

For enumeration of microorganisms from unknown samples all sample aliquots and dilutions will be analyzed in duplicate. Precision control charts will be maintained for each analyst and updated on a monthly basis by analyzing a minimum of twenty (20) replicates from a known positive sample. In addition, ten percent of all microbial analyses conducted in a participating laboratory will be performed by a second analyst.

4.5.3 Verification Checks

A minimum of ten (10) percent of all plate counts will be verified by a second analyst.

4.5.4 Confirmation Checks

A minimum of ten (10) percent of all analyses conducted on selective and/or differential media will be confirmed. For confirmation, at least ten (10) percent of all isolates from a sample should be confirmed. For specific confirmation tests consult the individual methods.

4.5.5 Sterility Checks

Sterility checks must be performed with each sample batch analyzed for enumeration. In addition, all media and reagent batches must be verified and documented for sterility.

TABLE 4.6 APPROVED METHODOLOGY FOR ANALYSIS OF MICROBIOLOGY PARAMETERS⁹

Parameter	Method	Description	Units Reported	Det'n Limit
Heterotrophic plate count ^a	SM 900	R2A, 28 C, 7days ^b	CFU/100 ML	1
Heterotrophic plate count ^a	SM 900	PCA, 28 C, 7days ^b	CFU/100mL	1
Non-saprophytic plate count ^c	TBD	CAE ^d , 35 C, 24 hr	CFU/100 ML	1
Fecal coliforms	SM 900	mFC, 44.5 C, 24 hr	CFU/100 ML	1
Yeast and Mold	TBD	Emmon's ^e 20 C, 5days	CFU/100 ML	1
Total Count	TBD	epifluorescence	Cells/ML approx. 10 ³	
Gram positive	TBD	PCA ^f , 28 C, 48 hr	CFU/100 ML	1
Gram negative	TBD	PCA ^g , 28 C, 48 hr	CFU/100 ML	1
Anaerobes ^h	TBD	Brewers Anaerobic Agar 28 C, 10 days	CFU/100 ML	1
Enteric	TBD	PCA ⁱ , 35 C, 24 hr	CFU/100 ML	1
Legionellae	TBD	BCYE ^j , 35 C, 5 days	CFU/100 ML	333

^a includes culturable aerobes and facultative anaerobes

^b incubation times may be extended up to 21 days

^c may be better identified as aerotolerant eutrophic mesophiles (AEMs)

^d Chocolate Agar enriched with X and V factors

^e Sabouraud Dextrose Agar with Rose Bengal and Chloramphenicol

^f Plate Count Agar with phenylethanol

^g Plate Count Agar with Crystal Violet

^h includes culturable obligate and facultative anaerobes

ⁱ Plate Count Agar with Bile Salts

^j supplemented with glycine and antibiotics

4.5.6 Analytical Control Samples

At the present time few analytical control samples exist for microbiological parameters. Table 4.7 lists the parameters for microbiological control samples planned for use as qualification, verification and reference samples during the Phase III activities. The qualification, verification and blind reference samples will be used as outlined in Sections 3.3. In addition, blind duplicate samples will be submitted to the participating laboratories for microbiological analysis. These may be sent to a single laboratory or split between two or more laboratories for analysis. In addition, each participating laboratory will be expected to participate in an approved interlaboratory control program such as the EPA laboratory monitoring program conducted by the Quality Assurance Branch, Environmental Monitoring Systems Laboratory located at Cincinnati, Ohio.

TABLE 4.7 Typical Microbiological Parameters for Qualification, Verification and Reference Check Samples

Parameter	Concentration	Notes
Fecal coliforms	$10^6 - 10^9$	see note a
Gram Negatives	$10^6 - 10^9$	see note b
Gram Positives	$10^6 - 10^9$	see note b
Heterotrophs	$10^6 - 10^9$	see note b
Identification	N/A	see note c

Note a includes 0-2 fecal coliforms for detection and/or enumeration

Note b includes Gram positive or Gram negative organisms for detection and enumeration

Note c includes 1 ATCC culture for isolation and identification

5.0 REQUIREMENTS FOR THE CHEMICAL ANALYSIS OF AIR

5.1 Sample Collection

The variables associated with field collection of air samples often affect the results more than the analytical procedures being used. Field sampling personnel should be familiar with sampling and measurement procedures and equipment to be used.

5.1.1 Physical Properties

The physical properties of the contaminant(s) being sampled are a major factor in determining the collection procedure to be employed. Physical properties which are important include boiling point, vapor pressure, polarity and solubility in water and organic solvents. Other factors affecting the collection of airborne contaminants include temperature, humidity, sampling flow rates, chemical properties, volatility and concentration of the contaminant(s). Each of these must be considered when developing a sampling method and strategy.

5.1.2 Volatility

Organic compounds may be classified based on their degree of volatility. Each of these classes are briefly described below.

5.1.2.1 Volatile Organic Compounds

Volatile organic compounds (VOCs) may be characterized as non-methane organic compounds having vapor pressures greater than 10^{-2} kPa. These compounds predominately occur as gasses at standard temperature and pressure and may be collected and concentrated by high volume sampling on solid sorbants. The

compounds are then thermally desorbed from the sorbant medium for analysis.

5.1.2.2 Semi-Volatile Organic Compounds

Semi-volatile organic compounds (SVOCs) represent a wide variety of organic contaminants which have vapor pressures which range from 10^{-2} to 10^{-8} kPa. SVOCs are present in air as gases and as condensed particle-bound constituents. Because of this sampling regimes for SVOCs must include distribution analysis for quantitative recovery.

5.1.2.3 Non-Volatile Compounds

Non-volatile compounds (NVCs) may be described as those compounds having vapor pressures less than 10^{-8} kPa. NVCs associated with ambient air are primarily recovered as condensed particle-bound constituents. The collection and concentration of NVCs from ambient air is usually accomplished by high volume sampling through particulate filters and subsequent extraction of the NVCs from the filter material.

5.1.3 Sampling Media

Various sampling media may be used for the collection and concentration of airborne organic contaminants. The sampling media will be specified for the parameter of interest by the method to be used. This may include specific filter types and porosity, concentration and volume of liquid media or amount and type of solid sorbent to be used. Most sampling media commonly used are well characterized. If specific products are specified no substitutions will be made without the prior written approval by the Principal Investigator, Medical Monitor and/or NASA/MSFC

Material and Processing Laboratory.

5.1.4 Sampling Equipment

Sampling equipment must be operated properly and maintained in good working condition. Periodic maintenance and inspection is a necessary function of the sampling personnel. Pumps should be chosen which are compatible with the sampling requirements outlined in a particular method. The pump must be capable of maintaining the recommended flow rates and sampling times required to meet the specified detection limits. All pumps must be calibrated prior to use with the representative sample train to be used in place. When practical, flow rates are measured during sample collection as well.

5.1.5 Recordkeeping

Accurate recordkeeping in the field is essential. All pertinent information including sample location, calibration information, sampling times, sampler, temperature, humidity, possible interfering compounds and any anomalies should be documented. The exact sampling time and flow rate are necessary to correctly estimate the volume of air sampled. This is accomplished using the initial calibration data, start and stop times and periodic spot checks to assure the pump is operating properly during the collection procedure.

5.1.6 Contamination

Since modern analytical techniques are extremely sensitive, special care must be taken to minimize contamination of field samples. Samples must not be stored or shipped with bulk

materials which can contaminate the sample. Glassware or other containers used in sampling and/or shipping should be cleaned and free of any contaminating materials. Field blanks are routinely used to estimate the contamination which may occur during sampling, transport and storage prior to analysis.

5.1.7 Sampling Protocol

A written sampling protocol will be developed and approved by the Principal Investigator prior to sample collection. The protocol should include a statement of purpose, description of the area to be sampled, number and location of sample sites, collection and analysis methods to be used, number and type of samples and blanks to be collected and analyzed and specific analytical controls to be employed.

5.2 Analytical Methods

Analytical methods to be used will be chosen from recognized sources. The primary source for the determination and quantification of organic contaminants from ambient air is the USEPA "Compendium of Methods for the Determination of Toxic Organic Compounds in Ambient Air".¹⁰ Secondary methods, if required, will be from the NIOSH "Manual of Analytical Methods" and/or APHA "Methods of Air Sampling and Analysis".^{11,12} Specific parameters of interest and approved methods are listed in Table 5-1.

TABLE 5-1. Recommended Methods for the Analysis of Selected Toxic Organic Contaminants In Ambient Air¹⁰

COMPOUND	APPLICABLE METHODS
Acetaldehyde	TO-5
Acrolein	TO-5
Acrylonitrile	TO-2, TO-3
Allyl Chloride	TO-2, TO-3
Aldrin	TO-4, TO-10
Benzaldehyde	TO-5
Benzene	TO-1, TO-2, TO-3, TO-14
Benzo (a) Pyrene	TO-13
Benzylchloride	TO-1, TO-3, TO-14
Bromoform (Tribromomethane)	TO-1, TO-3
Bromobenzene	TO-1
Bromomethane	TO-14
Carbon Tetrachloride	TO-2, TO-3, TO-14
Chlordane	TO-10
Chlorobenzene	TO-1, TO-3, TO-14
Chloroethane	TO-14
Chloromethane	TO-14
Chloroform	TO-1, TO-2, TO-3
Cresols (o, m, p - Methyl Phenols)	TO-8
Cumene	TO-1
2,4,-D esters	TO-10
4,4'-DDE	TO-4, TO-10
4,4'-DDT	TO-4, TO-10
1,2-Dichlorobenzene	TO-14
1,3-Dichlorobenzene	TO-14
1,4-Dichlorobenzene	TO-1, TO-3, TO-14
Dichlorodifluoromethane	TO-14
1,2-Dichloroethane	TO-1, TO-2, To-3
1,1-Dichloroethene	TO-2, TO-3, TO-14
<u>cis</u> -1,2-Dichloroethylene	TO-14
1,2-Dichloropropane	TO-1, TO-14
1,3-Dichloropropane	TO-1
<u>cis</u> -1,3-Dichloropropene	TO-14
<u>trans</u> -1,3-Dichloropropene	TO-14
1,2-Dichloro-1,1,2,2-tetrafluoroethane	TO-14
Dieldrin	TO-10
Endrin	TO-10
Endrin Aldehyde	TO-10
Ethylbenzene	TO-1, TO-14
Ethylene Dibromide	TO-1, TO-14
4-Ethyltoluene	TO-14

TABLE 5-1. Recommended Methods for the Analysis of Selected Toxic Organic Contaminants In Ambient Air¹⁰ (Continued)

COMPOUND	APPLICABLE METHODS
Formaldehyde	TO-5, TO-11
Heptachlor	TO-10
Heptachlor epoxide	TO-10
1,2,3,4,7,8-Hexachlorodibenzo-p-Dioxin	TO-9
Hexachlorobenzene	TO-10
Hexachlorobutadiene	TO-14
B-Hexachlorocyclohexanes	TO-10
Lindane	TO-10
Methoxychlor	TO-10
Methylene Chloride	TO-2, TO-3, TO-14
Nitrobenzene	TO-1, TO-3
N-Nitrosodimethylamine	TO-7
Octachlorodibenzo-p-Dioxin	TO-9
Oxychlordanes	TO-10
Pentachlorobenzene	TO-10
Pentachlorophenol	TO-10
Phenol	TO-8
Polychlorinated Biphenyls	TO-4
Propanal	TO-5
Styrene	TO-14
1,2,3,4-Tetrachlorodibenzo-p-Dioxin	TO-9
2,3,7,8-Tetrachlorodibenzo-p-Dioxin	TO-9
1,1,2,2,-Tetrachloroethane	TO-14
Tetrachloroethylene	TO-1, TO-3, TO-14
Toluene	TO-1, TO-2, TO-3, TO-14
1,2,4-Trichlorobenzene	TO-14
1,1,1-Trichloroethane	TO-2, TO-3, TO-14
1,1,2-Trichloroethane	TO-14
Trichlorofluoromethane	TO-14
1,1,2-Trichloro-1,2,2-trifluoroethane	TO-14
2,4,5-Trichlorophenol	TO-10
1,2,4-Trimethylbenzene	TO-14
1,3,5-Trimethylbenzene	TO-14
Vinyl Chloride	TO-2, TO-3, TO-14
o, m, p-Xylene	TO-1, TO-3, TO-14

5.3 Analytical Control Procedures

TBD

6.0 REQUIREMENTS FOR MATERIALS AND PROCESS CONTROLS

6.1 Sample Collection and Preparation

Samples will be collected by Boeing Environmental Laboratory (BAC) personnel according to the daily sampling schedule created by the MSFC Test Laboratory (EL-64) data custodian. Samples will be split under the direction of EL-64 personnel into appropriately prepared and preserved sample bottles. The collected samples will be transferred to the laboratory area where batch and sample numbers will be assigned, appropriate control samples added and the samples recorded into a Sample Log Book. All bottles and sample labels will be prepared and delivered to the EL-64 data custodian at least ⁴⁸~~24~~ hours prior to sampling. Figure 6.1 illustrates the logistics of sample collection activities.

6.2 Field Numbers

At the time of collection, samples will be logged into a Field Notebook and assigned a field number. The field notebook will remain with the samplers. Field numbers will be assigned as follows:

XX-AA-BB-CCC

Where,

XX = A sequentially numbered field log book

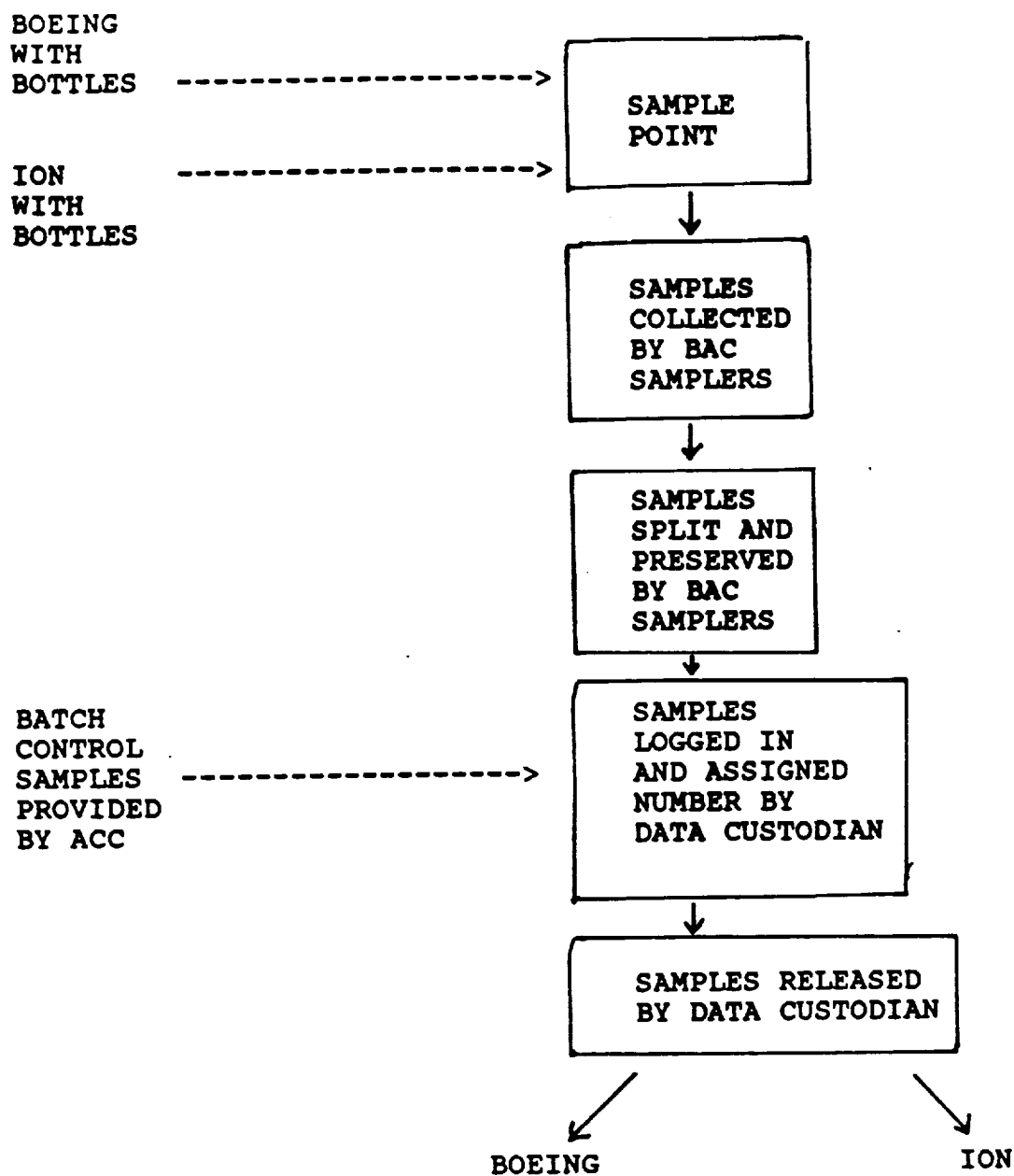
AA = The page that the sample is recorded on

BB = The entry of the sample on page AA

CCC = The initials of the sampler

The field number must be included on the sample label and chain-of-custody form for tracking purposes.

Figure 6.1 Sampling Collection and Logistics Flow Chart



6.3 Sample Labels

Sample collection labels will be prepared and affixed to each sample container. The sample labels should minimally contain the following information:

- o Sample collection number
- o Date collected
- o Time collected
- o Collection location/description
- o Initials of personnel collecting the sample
- o Parameters for analysis
- o How preserved
- ~~o Disinfection procedure used~~
- o Any anomalies encountered during sampling
- o Laboratory to which the sample is to be sent

An illustration of the sample label is provided as Figure 6-2.

6.4 Sample Log In

Immediately after collection, samples will be transferred to the Laboratory located in building 4755. At this point samples will be logged into a Master Log Book and assigned a permanent sample number. The log book will be maintained by the Data Custodian and will remain on-site at the Test Facility located at MSFC Building 4755. The sample numbers will consist of the test stage, test day, batch assignment, sample group and subgroup, sample port and laboratory assignment.

Example: XXX-AA-BB-C-DDDD-EE-F-GGG

Where,

- XXX = The test name (WRT)
- AA = The test Stage (1A,2B,3A, etc.)
- BB = The Test Day (1-99)
- C = The particular batch (1-4)
- DDDD = The analysis group and subgroup
- EE = The sample port
- F = The laboratory assigned to conduct the analysis
- GGG = The unit description

In addition, the following information should be included in the Sample Log Book:

- o Date Collected
- o Time Collected
- o Collected By (initials)
- o Sample Description
- o Analysis Group and Subgroup
- o Preservative
- ~~o Disinfection~~
- o Comments
- o Field Number (if applicable)

An example of the layout for the Sample Log is illustrated by Figure 6-3.

Figure 6.2

TBD

Sample Log In Book

To: _____

Sample Number	Sample Received By	Date/Time Received	Sample Description	Date/Time Collected	Collected By	Parameters	Comments	Test Complete

Figure 6-3

6.5 Electronic Data Entry

Electronic data entry has been suggested as an alternative to the manual log in procedure. In this case, data should be saved after every ten (10) entries. Additionally, duplicate backup of the Sample Log Book data will be made following each data entry session. One copy will be maintained at the test site at all times and the second copy will be in the custody of the Data Custodian.

6.6 Chain of Custody

At the time samples are recorded into the sample log book a chain of custody form is also completed for each sample batch. At this time the samples are relinquished to the ^{Data Custodian} ACC for distribution to the participating laboratories. A copy of the chain of custody form is illustrated in Figure 6-4.

6.7 Sample Storage

Samples will be segregated by batch and the participating laboratory to which the samples are to be sent. Samples will be stored at 4° C until relinquished by the Data Custodian. Samples will remain in MSFC Building 4755 for no more than six (6) hours after collection.

6.8 Analysis of Test Parameters

Test parameters will be analyzed simultaneously within the assigned batches. Results will then be summarized per individual samples and reported on a master data sheet (Figure 6-5). Analysis data will be reviewed by the ACC and compared to internal batch controls and parameters labeled as "suspect" or

"acceptable". All suspect data will be evaluated by a review committee headed by the NASA/MSFC Materials and Process Laboratory for final disposition.

6.9 Analysis of Control Samples

Results obtained from intralaboratory control samples should be submitted with those from the unknown samples. This data will be used by the review committee to make a final decision regarding the validity and/or limitations of "suspect" data.

BOILING LABORATORIES CHAIN OF CUSTODY RECORD

Figure 6-4

[illegible]

Figure 6-5

MASTER DATA SHEET

LABORATORY: _____ SAMPLE NUMBER: _____

DATE COLLECTED: _____ DATE RECEIVED: _____

COMMENTS: _____

PARAMETER	RESULTS	UNITS	DATE COMPLETED	STATUS
PHYSICAL				
Conductivity	_____	umhos	_____	_____
pH	_____		_____	_____
Total Solids	_____	mg/l	_____	_____
TDS	_____	mg/l	_____	_____
TSS	_____	mg/l	_____	_____
Inorganic Non-Metals				
Alkalinity	_____	mg/l	_____	_____
Ammonia	_____	mg/l	_____	_____
Chloride	_____	mg/l	_____	_____
Chlorine	_____	mg/l	_____	_____
Fluoride	_____	mg/l	_____	_____
Nitrate	_____	mg/l	_____	_____
TKN	_____	mg/l	_____	_____
Phosphate	_____	mg/l	_____	_____
Sulfate	_____	mg/l	_____	_____
Metals				
Arsenic	_____	ug/l	_____	_____
Barium	_____	ug/l	_____	_____
Cadmium	_____	ug/l	_____	_____
Calcium	_____	ug/l	_____	_____
Chromium	_____	ug/l	_____	_____
Copper	_____	ug/l	_____	_____
Iron	_____	ug/l	_____	_____
Lead	_____	ug/l	_____	_____
Magnesium	_____	ug/l	_____	_____
Manganese	_____	ug/l	_____	_____
Mercury	_____	ug/l	_____	_____
Nickel	_____	ug/l	_____	_____
Potassium	_____	ug/l	_____	_____
Selenium	_____	ug/l	_____	_____
Silver	_____	ug/l	_____	_____
Sodium	_____	ug/l	_____	_____
Zinc	_____	ug/l	_____	_____

6.10 Intralaboratory Data Handling and Reporting

The intralaboratory documentation system for data handling and reporting will be described. This would include all documentation and associated procedures for sample tracking. In addition, the flow process procedures for completion of data sheets for intralaboratory use should be defined and illustrated. Responsible personnel should be identified for each phase of the documentation system.

Report formats for collection and interpretation of unknown and control data should be described. Reported data should only contain significant figures. The following are guidelines¹ for determining significant figures:

(1) A value is made up of significant figures when it contains all digits known to be true and the last digit in doubt. For example, if a figure is reported as 18.8 mg/l, the 18 must be firm while the 0.8 is somewhat uncertain, but presumably better than one of the values 0.7 or 0.9 would be.

(2) Final zeros after a decimal point are always meant to be significant figures. For example, to the nearest milligram, 9.8 is reported as 9.800 g.

(3) Zeros before a decimal point with nonzero digits preceding them are significant. With no preceding nonzero digit, a zero before the decimal point is not significant.

(4) If there are no nonzero digits preceding a decimal point, the zeros after the decimal point but preceding other nonzero digits are not significant. These zeros only indicate the position of the decimal point.

Proper use of significant figures gives an indication of the reliability of the analytical method used. Analysts should take care not to report a result with more digits than are significant.

The following rules¹ for rounding numbers will apply to all reported data:

(1) Rounding off of numbers is a necessary operation in all analytical areas. It is automatically applied by the limits of measurement of every instrument and all glassware. However, when it is applied in chemical calculations incorrectly or prematurely, it can adversely affect the final results.

(2) If the figure following those to be retained is less than 5, the figure is dropped, and the retained figures are kept unchanged. As an example, 11.443 is rounded off to 11.44.

(3) If the figure following those to be retained is greater than 5, the figure is dropped, and the retained figure is raised by one. As an example, 11.446 is rounded off to 11.45.

(4) If the figure following those to be retained is 5, and if there are no figures other than zeros beyond the five, the figure 5 is dropped, and the last-place figure retained is increased by one if it is an odd number or it is kept unchanged if an even number. As an example, 11.435 is rounded off to 11.44, while 11.425 is rounded off to 11.42.

(5) When a series of numbers is added, the sum should be rounded off to the same number of decimal places as the addend with the smallest number of places. However, the operation is completed with all decimal places intact, and the rounding off is done afterward.

(6) When one number is subtracted from another, rounding off should be completed after the subtraction operation, to avoid possible invalidation of the operation.

(7) When two numbers are to be multiplied, all digits are carried through the operation, then the product is rounded off to the number of significant digits of the multiplier with the fewer significant digits.

(8) When two numbers are to be divided, the division is carried out on the two numbers using all digits. Then the quotient is rounded off to the number of significant digits of the divisor or dividend, whichever has the fewer.

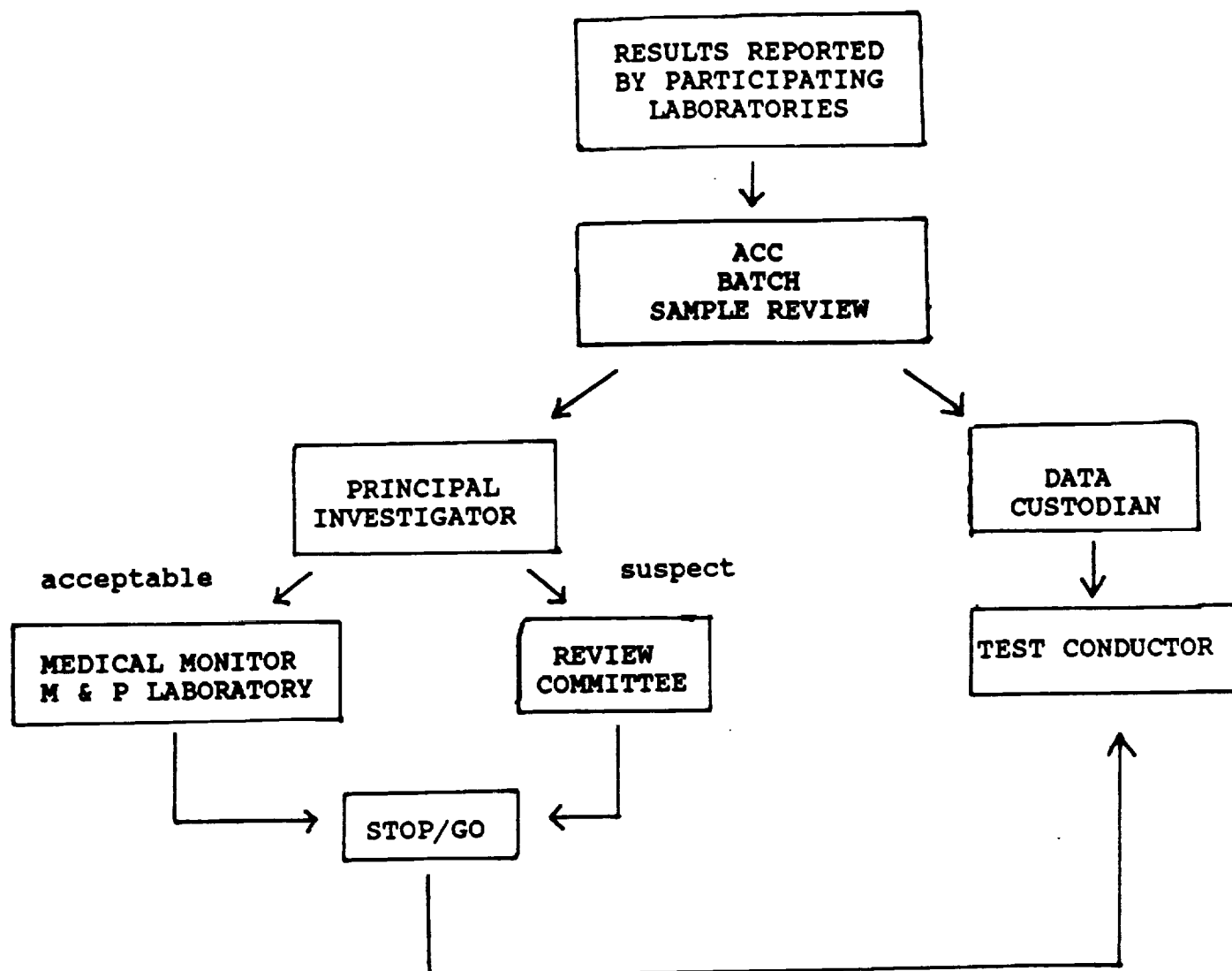
(9) When a number contains n significant digits, its root can be relied on for n digits, but its power can rarely be relied on for n digits.

The preceding rules for rounding off are reasonable for most calculations; however, when dealing with two nearly equal numbers, there is a danger of loss of significance when applied to a series of computations that rely on a relatively small difference in two values. Examples are calculation of variance and standard deviation. The recommended procedure is to carry several extra figures through the calculations and then to round off the final answer to the proper number of significant figures.

6.11 Interlaboratory Data Handling and Reporting

Laboratory results will be reported directly to the ACC on the Master Data Sheet (Figure 6-5). Upon review by the ACC the data sheets will be forwarded to the Principal Investigator. In turn, the Principal Investigator will immediately forward a copy of all results obtained from sample groups A and B to the Medical Monitor. In addition, a copy of all data will be sent to the data custodian. Figure 6-6 illustrates the flow of data from the participating laboratories.

Figure 6-6 Data Reporting Flow Chart



7.0 SAFETY

Each participating laboratory should have a written Laboratory Safety document. As a minimum the Laboratory Safety document should address the following:

- o General Laboratory Guidelines
- o Fire and Emergency Procedures
- o Chemical Hazards
- o Biological Hazards
- o Radiological Hazards
- o Reporting of incidents and accidents
- o Emergency Medical Care
- o Safety Training
- o Log of personnel who has read the document

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